SUPPORT FOR THE AMENDMENTS

Newly-added Claims 52-66 are supported by the specification at and the original claims. Accordingly, no new matter is believed to have been added to the present application by the amendments submitted above.

REMARKS

Claims 52-66 are pending.

Applicants would like to thank Examiner Zara fro indicating that the oligomeric sequence AACGTTAT is free of the prior art. See page 7 of the Office Action. Favorable reconsideration is respectfully requested.

The present invention relates to an antitumor composition comprising an immunostimulatory oligonucleotide comprising a nonmethylated octameric CG motif of the sequence AACGTTAT. See Claim 52.

The present invention also relates to an oligonucleotide comprising a nonmethylated octameric CG motif of the sequence AACGTTAT. See Claim 63.

The rejections of the Claims under 35 U.S.C. §112, first paragraph, are believed to be obviated by the amendment submitted above in part and is, in part, respectfully traversed.

As discussed above, the invention is directed to an immunostimulatory oligonucleotide comprising a nonmethylated octameric CG motif of the sequence AACGTTAT. See Claims 52 and 63.

The present specification provides not only a disclosure of an antitumor activity for the oligonucleotide of the sequence SED ID NO:2, but also provides evidence that several oligonucleotides having the octameric motif AACGTTAT (An15 (SEQ ID NO:9): example 13 and figure 10, example 12 and figure 7; An21 (SEQ ID NO:10): example 13 and figure 9) have enhanced activity compared to other nucleotides not having this motif (An14 (SEQ ID NO:3): figure 10). Furthermore, the specification provides a detailed analysis of the effect of the sequence of the octameric motif on the antitumor activity (example 13: figures 8 to 10 and 11); not less than 12 oligonucleotides having a different octameric motif were compared. The results presented in figures 8 to 10 and 11 clearly show that the octameric motif

AACGTTAT has enhanced activity compared to other motif not having AT adjacent to AACGTT (An23 (AC), An24(AG), An26(GT), An28(CC), PT1(CG); figure 11).

In addition, example 12 (figure 7) shows the antitumor activity for the different stabilized oligonucleotides (phosphorothioate (PT), phosphodiester (PDE), methylphosphonate (MP), phosphodiester-phosphorothioate mixture (mixed) and stabilization at one end (3').

Regarding the comment in the Office Action at page 4, lines 6-8, the specification describes the incorporation of 5-bromocytosine in the nonmethylated octameric CG motif of the sequence AACGTTAT. See page 7, lines 7 to 10 of the specification.

The suggestion in the Office Action page 5, lines 3 to 15, that the length of the oligonucleotides is a rate-limiting step for achieving an efficient delivery of the oligonucleotides in the cancer cells and thus providing a therapeutic effect is based upon published literature concerning antisense oligonucleotides (Agrawal et al., Mol. Med. Today, 2000, 6, 72-81; Chirila et al., Biomaterials, 2002, 23, 321-342). However, that suggestion is not pertinent for the immunostimulatory oligonucleotides of the claimed methods, which are different from the antisense oligonucleotides of the cited reference. In fact, the immunostimulatory properties of these oligonucleotides were discovered by using large DNA molecules [DNA fraction from *Mycobacterium bovis* BCG; Tokunaga et al., *J. Natl., Cancer Inst.*, 1984, 72, 955-962 (abstract submitted herewith)]. BCG DNA preparation have clinical applications as immunostimulant for the treatment of bladder cancers [Soloway et al., *Urol., Clin., North Am.*, 1988, 15, 661-669 (abstract submitted herewith)]. It was shown that the immunostimulatory properties are dependent on nonmethylated CpG dinucleotides (nonmethylated CG motif) which are under-represented in mammalian DNA [Yamamoto et al., *J. Immunol.*, 1992, 148, 4072-4076 (abstract submitted herewith); Krieg et al., *Nature*, 1995, 374, 546-549 (submitted herewith)]. It is well-known in the art that both large DNA

molecules (bacterial DNA; several kilobases) and synthetic oligodeoxynucleotides (ODNs; 15-30 bases) containing said CpG dinucleotides are equally effective at inducing an immune activation (Krieg et al., *Nature*, 1995, 374, 546-549). This was confirmed recently by the demonstration that CpG DNA (large DNA and ODNs) acts through binding to the TLR9 receptor in the early endosome and transduction of the intracytoplasmic signal by TLR9 (see for example figure 3 of Takeshita et al., Seminars in Immunology, 2004, 16, 17-22 (submitted herewith). Thus, a therapeutic effect is achieved with CpG DNA of any length: oligodexoynucleotides produced by chemical synthesis (15-30 bases), plasmids or viral DNA (several kiolobases), produced by recombinant DNA technology.

The present specification describes that CpG-ODNs have a therapeutic effect against different tumor types (glioma (examples 1 to 8, 12, 13)); neuroblastoma (examples 9 to 11), *in vivo*, in immuno-competent animal models; local (intratumoral) or systemic (subcutaneous or intraperitoneal) administration of these CpG-ODNs produces a significant reduction in the tumor volume as compared with control oligonucleotides, in both tumor types.

The published literature using immunostimulatory oligonucleotides comprising nonmethylated CG motifs as single agents demonstrates that systemic or local CpG oligonucleotides administration alone can induce tumor regression established tumors, in various models of cancer [reviewed in Carpentier et al., *Front. Biosci.*, 2003, 8, 115-127, (abstract submitted herewith)].

In addition, preclinical models for the treatment of human cancers by administration of CpG ODNs have shown impressive results and several clinical trials are on-going worldwide in melanoma, lymphoma, renal carcinoma, breast cancer and glioblastoma (see the abstracts of the 2004 ASCO Annual Meeting, Carpentier et al., Neeuro-Oncology, 2006, 8, and Friedberg et al., Blood, 2005, 105, 489-495 (submitted herewith)).

In the animal model (rat) presented in the present specification, the antitumor activity is observed with a dose of 50 μg of oligonucleotide (example 12, for a tumor with a diameter of 10 mm, tumor volume of about 0.5 ml); this corresponds to 1 mg of oligonucleotide for a tumor mass of 10 ml. In a human, the tumor mass is from 10 ml to about 100 ml, which corresponds to a dose of about 1 to 10 mg of oligonucleotide. These doses are in the range of those used in the clinical trials for the treatment of melanomas by local injection of CpG oligonucleotides.

Therefore, the detailed teaching of the present specification in combination with the general knowledge at the time the invention was made provides the skilled artisan with the information necessary to practice the claimed method.

In view of the foregoing, withdrawal of these grounds of rejection is respectfully requested.

Regarding sequence compliance, Claim 48 has been canceled and the subject matter of those claims is not recited in the newly-added claims.

Regarding the Restriction Requirement, the non-elected claims have been canceled.

Applicants submit that the present application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

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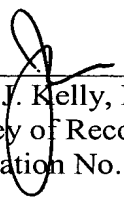
Norman F. Oblon



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A1

1: J Natl Cancer Inst. 1984 Apr;72(4):955-62.

Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis BCG. I. Isolation, physicochemical characterization, and antitumor activity.

Tokunaga T, Yamamoto H, Shimada S, Abe H, Fukuda T, Fujisawa Y, Furutani Y, Yano O, Kataoka T, Sudo T, et al.

A fraction extracted from Mycobacterium bovis strain BCG, which was composed of 70.0% DNA, 28.0% RNA, 1.3% protein, 0.20% glucose, and 0.1% lipid and of no detectable amounts of cell wall components such as alpha, epsilon-diaminopimelic acid and hexosamine, was found to possess strong antitumor activity. Repeated intralesional injection of this fraction, designated MY-1, without attachment to oil or a single intralesional injection of MY-1 emulsified in mineral oil caused the IMC carcinoma of CDF1 mice and line 10 tumor of strain 2 guinea pigs to regress and/or prevented metastasis very effectively. MY-1 after digestion with RNase, which contained 97.0% single-stranded DNA with a guanine-cytosine content of 69.8%, was more effective than undigested MY-1 against IMC and line 10 tumor, while MY-1 digested with DNase, which contained 97.0% RNA, had reduced activity, suggesting that the DNA from BCG possessed strong antitumor activity under certain conditions. Details of the extraction procedures and physicochemical characterization of MY-1 were also described.

PMID: 6200641 [PubMed - indexed for MEDLINE]

1: Urol Clin North Am. 1988 Nov;15(4):661-9.

Intravesical therapy for bladder cancer.

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Bladder tumors confined to the mucosa and lamina propria are heterogeneous. Papillary, low-grade (I-II) noninvasive tumors (Ta) may recur frequently, thus subjecting the patient to numerous endoscopic procedures, but these patients infrequently have progression in grade or stage. Treatment need not be overly aggressive. High-grade (III) tumors confined to the mucosa (carcinoma in situ or Ta) or with lamina propria invasion (T1) require not only thorough initial endoscopic resection if possible, but extremely careful monitoring. Intravesical therapy should be seriously considered, as a subsequent tumor might invade the muscle and thus be life threatening. At this writing, there are no conclusive data to indicate which intravesical agent is most appropriate for each circumstance. Randomized trials are in progress. A few statements are possible, however. Thiotepa delays the development of low-grade tumors when used for prophylaxis. Toxicity is low, and the drug is not expensive. Mitomycin C is effective for treatment of residual superficial tumor and when instilled regularly after complete transurethral resection (prophylaxis). Side effects are infrequent (cystitis, dermatitis, rash) and almost never severe. The drug is expensive. Adriamycin appears to be active in the treatment and prophylaxis of superficial tumors, but its precise role has not been defined. Cystitis is common. BCG may be the most effective intravesical agent in the treatment of carcinoma in situ. The frequency and severity of local and systemic side effects vary with the strain but are potentially worse than with the chemotherapeutic agents. Thus, the clinician must carefully consider which patients to treat with BCG.

Publication Types:
- Review

PMID: 3142129 [PubMed - indexed for MEDLINE]

1: J Immunol. 1992 Jun 15;148(12):4072-6.

Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN [correction of INF] and augment IFN-mediated [correction of INF] natural killer activity.

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Thirty-mer single-stranded oligonucleotides, with a sequence chosen from the known cDNA encoding the 64-kDa protein named Ag A or the MPB-70 protein of Mycobacterium bovis BCG and the human cellular proteins such as complement component 1 inhibitor and Ig rearranged lambda-chain, were used to dissect the capability to induce IFN and to augment NK cell activity of mouse spleen cells by coincubation in vitro. Three with the hexamer palindromic sequence as GACGTC were active, whereas two kinds of oligonucleotides with no palindrome were inactive. The oligonucleotides containing at least one of the different palindromic sequences showed no activity. When a portion of the sequence of the inactive oligonucleotides was substituted with either palindromic sequence of GACGTC, AGCGCT, or AACGTT, the oligonucleotide acquired the ability to augment NK activity. In contrast, the oligonucleotides substituted with another palindromic sequence such as ACCGGT was without effect. Furthermore, exchange of two neighboring mononucleotides within, but not outside, the active palindromic sequence destroyed the ability of the oligonucleotides to augment NK cell activity. Stimulation of spleen cells with the substituted oligonucleotide, A4a-AAC, induced production of significant amounts of IFN-alpha/beta and small amounts of IFN-gamma. Augmentation of NK activity of the cells by the oligonucleotide was ascribed to IFN-alpha/beta production. These results strongly suggest that the presence of the unique palindromic sequences, such as GACGTC, AGCGCT, and AACGTT, but not ACCGGT, is essential for the immunostimulatory activity of oligonucleotides.

PMID: 1376349 [PubMed - indexed for MEDLINE]

overeating associated with certain obesity syndromes[14]. Moreover, 5-HT_{2C}R transcripts have been detected in the paraventricular nucleus of the hypothalamus[7], lesions of which result in a behavioural obesity syndrome[18]. Thus, 5-HT_{2C}R mutant mice differ from other rodent models of obesity such as *ob/ob* and *db/db* mice[25,26], which are characterized primarily by metabolic dysregulation. 5-HT_{2C}R deficient mice therefore provide a novel tool for elucidating defects in neurochemical pathways that underlie the abnormal control of appetite observed in mood and eating disorders.

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CpG motifs in bacterial DNA trigger direct B-cell activation

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UNMETHYLATED CpG dinucleotides are more frequent in the genomes of bacteria and viruses than of vertebrates. We report here that bacterial DNA and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides induce murine B cells to proliferate and secrete immunoglobulin *in vitro* and *in vivo*. This activation is enhanced by simultaneous signals delivered through the antigen receptor. Optimal B-cell activation requires a DNA motif in which an unmethylated CpG dinucleotide is flanked by two 5′ purines and two 3′ pyrimidines. Oligodeoxynucleotides containing this CpG motif induce more than 95% of all spleen B cells to enter the cell cycle. These data suggest a possible evolutionary link between immune defence based on the recognition of microbial DNA and the phenomenon of 'CpG suppression' in vertebrates. The potent immune activation by CpG oligonucleotides has implications for the design and interpretation of studies using 'antisense' oligonucleotides and points to possible new applications as adjuvants.

In addition to antigen-specific immune defences, lymphocytes, phagocytes and the complement system are directly activated by several microbial products including formyl methionine, teichoic acid, peptidoglycan, exopolysaccharides and lipopolysaccharide (LPS) (reviewed in ref. 1). Bacterial DNA also induces murine B-cell proliferation and immunoglobulin production, whereas mammalian DNA does not (Fig. 1*a* and ref. 2). In considering explanations for the mitogenicity of bacterial DNA, we noted that CpG dinucleotides generally are present at the expected frequency of 1 per 16 dinucleotides in microbial DNA, but they are only about one-quarter as prevalent in vertebrates[3]. In addition to this 'CpG suppression', the cytosines in CpG dinucleotides are highly methylated in vertebrates, but not microorganisms[3].

Methylation of bacterial DNA with CpG methylase abolished mitogenicity, demonstrating that this difference in CpG status is the cause of B-cell stimulation by bacterial DNA (Fig. 1*a*). The stimulatory effects of bacterial DNA were reproduced with synthetic oligodeoxynucleotides (oligonucleotides) that contain one or more CpG dinucleotides (Table 1). B cells isolated by flow cytometry to greater than 99% purity showed greater or equal activation to that represented in Table 1, demonstrating that accessory cells were not required. Oligonucleotides lost stimulatory activity if the CpG was eliminated (Table 1; compare oligonucleotide 1 to 1a; 2 to 2a, 3D to 3Dc; and 3M to 3Ma); if the oligonucleotide length was reduced below 8 bases (Table 1, oligonucleotide 2 versus 2e); or if the cytosine of the CpG was replaced by 5′-methylcytosine (Table 1; oligonucleotides 1b, 3Dd and 3Mb). In contrast, methylation of other cytosines (oligonucleotides 3De and 3Mc), or of CpG close to the end of an oligonucleotide (oligonucleotide 1c), did not reduce activity.

Optimal B-cell activation was seen with oligonucleotides containing a CpG motif in which the CpG dinucleotide was flanked by two 5′ purines (preferably a GpA dinucleotide) and two 3′ pyrimidines (preferably a TpC or TpT dinucleotide). Oligonucleotides in which bases were switched to bring the CpG motif closer to this ideal showed improved stimulation (for example, oligonucleotide 3M versus 3Md) whereas substitutions that disturbed the motif reduced stimulation (for example, compare oligonucleotide 2 to 2b, 2c and 2d or 3D to 3Df). Substitutions outside this CpG motif had little effect on stimulation (compare oligonucleotide 1 to 1d; 3D to 3Dg; 3M to 3Me). These data confirm that a CpG motif is the essential element present in oligonucleotides that activate B cells.

Kinetic studies demonstrated that B-cell activation was detectable by 4 h, peaked at 24 h, and returned toward baseline by 48 h (Fig. 1*b*). Cell-cycle analysis demonstrated that CpG oligonucleotides induce more than 95% of B cells to enter the cell cycle; a greater percentage than that induced by optimal LPS concentrations (Table 2). We also found that CpG oligonucleotides stimulate natural killer (NK) activity and protect B-cell

lines, against anti-IgM-induced growth inhibition (A.M.K. *et al.*, manuscripts in preparation).

CpG oligonucleotides with a nuclease-resistant phosphorothioate-modified backbone were synthesized to determine whether they would also cause immune stimulation. Indeed, phosphorothioate CpG oligonucleotides stimulated B cells at concentrations more than 2 logs lower than those required with unmodified oligonucleotides (Fig. 1*c*). Mice injected intraperitoneally with phosphorothioate CpG oligonucleotides had significant three- to sixfold increases in spleen B-cell proliferation and immunoglobulin secretion within 24 h ($P < 0.01$, not shown) and increased expression of activation markers such as class II major histocompatibility complex (MHC; Fig. 1*d*).

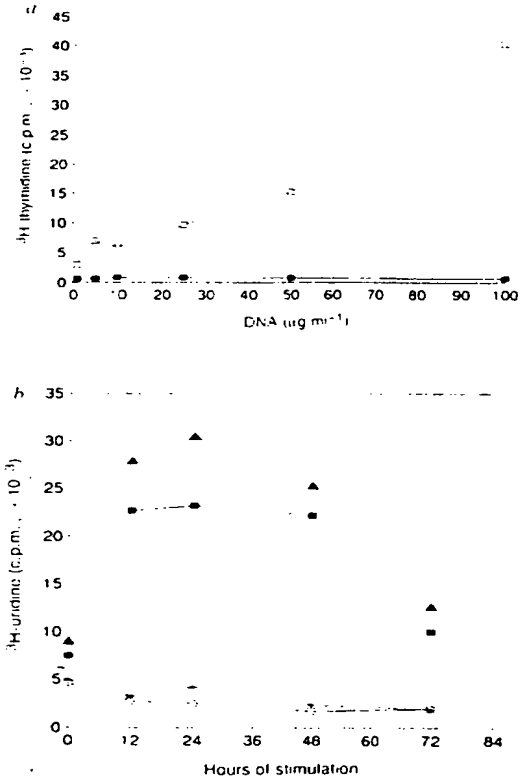
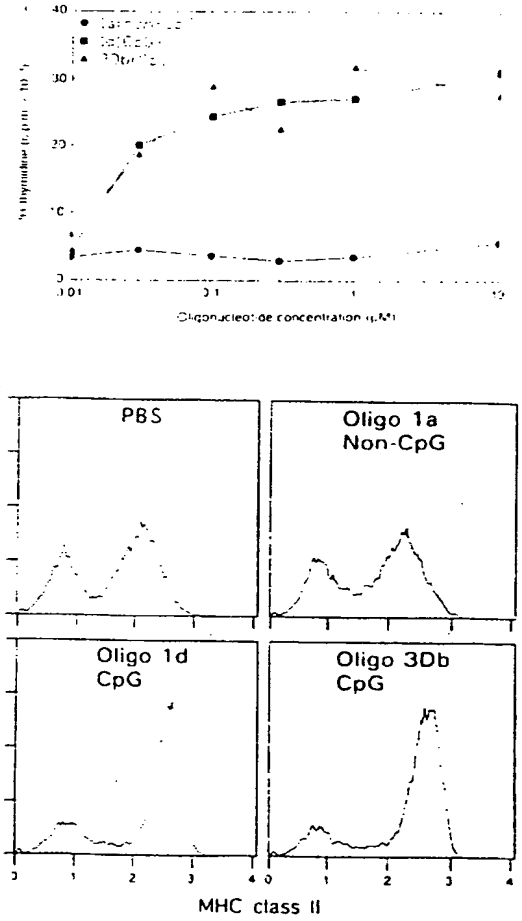


FIG. 1 *a*, B-cell stimulation by bacterial DNA requires unmethylated CpG dinucleotides. DBA 2 B cells (prepared as described in Table 1) were cultured with the indicated concentrations of heat-denatured (single-stranded) *E. coli* genomic DNA (which was 10–30% more mitogenic than dsDNA) for 44 h, then pulsed with ^{3}H-thymidine for 4 h before cell collection. The s.d. of the samples was <10%. Where indicated, *E. coli* DNA was methylated with 2 U CpG methylase (New England Biolabs) per μg DNA for 18 h at 37 °C (filled squares). Methylated DNA was completely protected against digestion with *Hpa*II but not *Msp*I. Bacterial DNA digested with DNAse I for 30 min at 37 °C was non-mitogenic. Unmethylated *E. coli* DNA (open squares) contained 2.5 ng of LPS per μg, and methylated DNA contained 12.5 ng LPS per μg by Limulus assay; both of which are below the LPS concentration which causes B-cell stimulation in our assay. Spleen B cells from C3H *HeJ* mice cultured with unmethylated *E. coli* DNA showed a similar dose response. As reported by Messina *et al.*[2] genomic DNA from NFS or NZB·N mouse liver caused minimal stimulation at any concentration (not shown). Unmethylated *E. coli* DNA induced an 8.8-fold increase in the number of IgM-secreting B cells by 48 h using the ELIspot assay described in Table 1. *b*, Time course of oligonucleotide stimulation of ^{3}H-uridine uptake. DBA 2 spleen B cells were cultured with either no oligonucleotide (open squares), or the CpG oligonucleotides 1 (filled triangles) and 2 (filled squares) or control oligonucleotide 1a (star) as described in Table 1 except that the cells were pulsed with ^{3}H-uridine at the time indicated on the *x*-axis and collected 4 h later. *c*, Dose response of oligonucleotides for B-cell stimulation. DBA 2 spleen B cells were

Antigen-specific B-cell activation requires both antigen binding to surface immunoglobulin and one or more costimulatory signals. To determine whether CpG oligonucleotides could provide costimulatory signals, primary B cells were cultured with or without CpG oligonucleotide and/or a suboptimal dose of α-IgM, which activates B cells by crosslinking surface immunoglobulin. B cells exposed to both CpG oligonucleotide and α-IgM showed marked synergistic proliferation (Fig. 2*a*). CpG oligonucleotides also synergistically activated antigen-specific immunoglobulin secretion in the mouse B-cell line CH12.LX, the antigen receptor of which binds sheep red blood cells (SRBC) (Fig. 2*b*). These data indicate that the CpG motif preferentially



cultured as described in Table 1 with phosphorothioate-modified oligonucleotides with the CpG sequences 1d (squares) or 3Db (triangles); or the control sequence 1a (circles). CpG oligonucleotides with nuclease-resistant methylphosphonate internucleotide linkages induced less mitogenic effect (not shown). *d*, *In vivo* immune activation by CpG oligonucleotides. Mice were injected i.p. with 0.25 ml of sterile PBS or the indicated phosphorothioate oligonucleotide (sequences as shown in Table 1) dissolved in PBS at a dose of 500 μg per mouse, which has been reported to give levels in spleen tissue of ~10 μg g^{-1} (1.54 μM)[3]. Doses below 100 μg per mouse gave minimal stimulation. Twenty-four hours later, spleen cells were collected, washed and stained for flow cytometry using anti-class II MHC (M5 114). The peak on the left represents spleen T cells, which did not change in number, but became a smaller percentage of the total population because of B-cell proliferation. There were similar increases in expression of the B-cell activation markers Ly-6A E, B7-2, Bla-1 and IgM (not shown). Two mice were studied for each condition and analysed individually. The experiment is one of four using several different mitogenic CpG oligonucleotides and six non-CpG oligonucleotides, which were all non-mitogenic.

activates B cells that simultaneously encounter their specific antigen.

The molecular mechanism by which CpG motifs induce B-cell activation is yet unclear. B cells do not appear to have a CpG-specific membrane receptor because there is no difference in their binding of fluorescent-tagged CpG and non-CpG oligonucleotides. CpG oligonucleotides linked to a solid support are non-stimulatory, indicating that cell uptake is required for activation (not shown). Double-stranded DNA is taken up by lymphocytes into an endosomal compartment where it is acidified and degraded to oligonucleotides over several hours[4]. Single-stranded oligonucleotides also appear to be taken up by B cells into an endosomal compartment[5], indicating that both double-stranded DNA and single-stranded oligonucleotides may have the same intracellular locations and forms.

These results demonstrate a new T-cell-independent pathway of B-cell activation triggered by a CpG motif common in microbial but not vertebrate DNA. By providing a costimulatory signal to B cells that bind antigen, microbial DNA may aid the emergence of specific antimicrobial immune responses. This ability of the vertebrate immune system to be specifically activated by microbial DNA may confer a selective advantage by improving the host response to infection. These data also suggest a possible pathogenic role for the increased levels of hypomethylated CpG sequences reported in patients with the autoimmune disease systemic lupus erythematosus[6,9].

The origin of CpG suppression is unclear[10]. We propose that CpG suppression and/or methylation may have been selected for during vertebrate evolution so that immune defences might

TABLE 1 B-cell stimulation by CpG oligonucleotides

Oligonucleotide	Sequence*	Stimulation index ³H Uridine†	Stimulation index IgM production‡
1	GCTAGACGTTAGCGT	6.1 ± 0.8	17.9 ± 3.6
1a	..... T	1.2 ± 0.2	1.7 ± 0.5
1b	..... Z	1.2 ± 0.1	1.8 ± 0.0
1c	..... Z.	10.3 ± 4.4	9.5 ± 1.8
1d	..AT.. ..AGC.	13.0 ± 2.3	18.3 ± 7.5
2	TCAACGTT	6.1 ± 1.4	19.2 ± 5.2
2a	.... GC..	1.1 ± 0.2	1.5 ± 1.1
2b	... G.. T.	4.5 ± 0.2	9.6 ± 3.4
2c	... T..A.	2.7 ± 1.0	ND
2d	.. TC..AA	1.3 ± 0.2	ND
2e	.....	1.3 ± 0.2	1.1 ± 0.5
3D	GAGAACGCTGGACGCTCGAT	4.9 ± 0.5	19.9 ± 3.6
3Da	..... T	6.6 ± 1.5	33.9 ± 6.8
3Db	..... C ..... G..	10.1 ± 2.8	25.4 ± 0.8
3Dc	... T.A	1.0 ± 0.1	1.2 ± 0.5
3Dd	..... C	1.2 ± 0.2	1.0 ± 0.4
3De	..... C	4.4 ± 1.2	18.8 ± 4.4
3Df	..... A	1.6 ± 0.1	7.7 ± 0.4
3Dg	..... GC. G.ACG..	6.1 ± 1.5	18.6 ± 1.5
3M	TCGATGTCGGTCCTGATCGT	4.1 ± 0.2	23.2 ± 4.9
3Ma	..... CT	0.9 ± 0.1	1.8 ± 0.5
3Mb	..... C	1.3 ± 0.3	1.5 ± 0.6
3Mc	..... C	5.4 ± 1.5	8.5 ± 2.6
3Md	..... A T	17.2 ± 9.4	ND
3Me	..... C..A.	3.6 ± 0.2	14.2 ± 5.2
LPS (30 μg ml⁻¹)		7.8 ± 2.5	4.8 ± 1.0

The oligonucleotides shown are representative of more than 300 studied including: two previously reported as 'antisense' oligonucleotides 3D and 3M: ref. 22); 48 8-base oligonucleotides; and 14 shorter oligonucleotides. CpG dinucleotides are underlined. Dots indicate identity. Z indicates 5-methylcytosine (Glen Research, Sterling, VA). Essentially identical results were obtained with oligonucleotides purchased from Operon Technologies (Alameda, CA) and from the DNA core facility, University of Iowa.

† Effects of oligonucleotides on B-cell RNA synthesis (as a measure of cell activation) were assessed using spleen cells from 6–12-week-old specific pathogen-free DBA 2 or BXSB mice that were depleted of T cells with anti-Thy-1.2 and complement and centrifugation over lympholyte M (Cedarlane Laboratories, Hornby, Ontario, Canada) as described[22]. Stimulation assays with 1 μCi of ³H uridine were done in 96-well microtitre plates with 20 μM oligonucleotide for 20 h. Similar stimulation was seen using C3H HeJ B cells, demonstrating that the mitogenic effects were not due to LPS contamination, and using SJL/J B cells, which are hyporesponders to the B-substituted guanosine B-cell mitogens. ³H-thymidine incorporation assays showed similar results, but with some nonspecific inhibition by thymidine released from degraded oligonucleotide[23]. CpG-oligonucleotides did not induce detectable tyrosine phosphorylation of B-cell proteins, inositol trisphosphate generation, or Ca^{2+} mobilization within 10 min. Oligonucleotides caused no detectable proliferation of γδ or other T-cell populations.

‡ Resting B cells (<0.02% T-cell contamination) were isolated from spleen cells treated with anti-Thy1, anti-CD4, anti-CD8 and complement[24] from the 63–70% band of a discontinuous Percoll gradient[25]. These were cultured as described above in 30 μM oligonucleotide or 20 μg ml⁻¹ LPS for 48 h. The number of IgM-secreting B cells was determined by ELIspot assay[26]. The background number of spots in unstimulated wells was 50 · 10^6 to 60 × 10^6 B cells. All assays were done in triplicate at least three times.

ND, Not done.

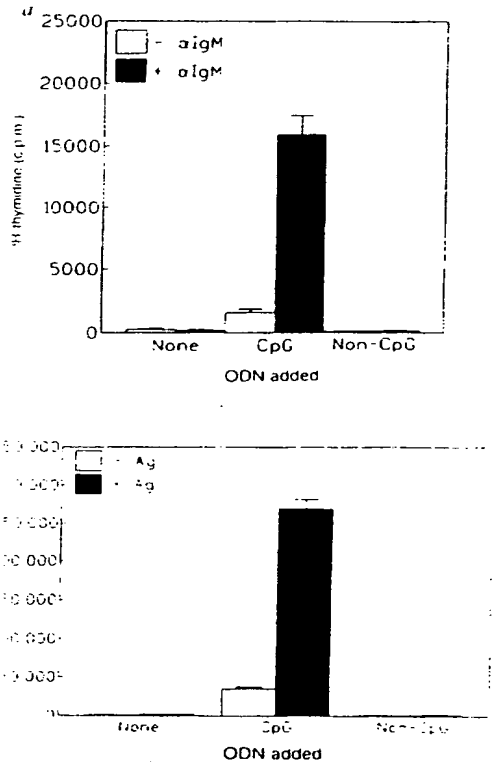


FIG. 2 Synergistic B-cell activation by CpG oligonucleotide and anti-IgM or antigen. a, Spleen B cells were prepared as in Table 1 and cultured with a monoclonal anti-IgM Ab (B-7-6, 1 μg ml⁻¹) and or phosphorothioate CpG oligonucleotide 1d or control oligonucleotide 1a at 0.1 μM and ³H-thymidine incorporation measured after 48 h. The experiment was done three times with similar results. b, CH12.LX cells were cultured as previously described[29] for 72 h with or without antigen (0.1% SRBC) and or 1 μg ml⁻¹ (~0.2 μM) of phosphorothioate-modified oligonucleotide 1d (CpG) or oligonucleotide 1a (Non-CpG). CH12.LX cells are not induced to secrete immunoglobulin by SRBC alone. IgM-secreting cells were enumerated as direct plaque-forming cells in a lawn of SRBC, as previously described[29]. Plaque-forming cells are measured per 10^6 viable recovered cells. Results are mean ± s.e.m. of duplicate cultures, and are representative of three experiments.

TABLE 2 Cell-cycle analysis with CpG oligonucleotides

Treatment	Percentage of cells in: G0	G1	S + G2 + M
Media	97.6	2.4	0.02
Oligonucleotide 1a (non-CpG)	95.2	4.8	0.04
Oligonucleotide 1d (CpG)	2.7	74.4	22.9
Oligonucleotide 3Db (CpG)	3.5	76.4	20.1
LPS (30 μg ml^{-1})	17.3	70.5	12.2

B cells (2×10^6) were cultured for 48 h in 2 ml tissue culture medium alone, or with 30 μg ml^{-1} LPS or with the indicated nuclease-resistant phosphorothioate oligonucleotide sequence at 1 μM. Cell-cycle analysis was as described[27]. The results shown are representative of three experiments using several different CpG and non-CpG oligonucleotides.

be triggered preferentially by microbial DNA. This hypothesis predicts that mitogenic CpGs may be more suppressed than non-stimulatory CpGs. Indeed, among human coding DNA sequences in GenBank, the frequency of the four most stimulatory hexamers (GACGTC, GACGTT, AACGTC and AACGTT) is $0.72 \pm 0.13 \times 10^{-4}$, less than one-third that of the four predicted most unfavourable CpG-containing sequences, (TCCGGA, TCCGGG, CCCGGA and CCCGGG), which was $2.22 \pm 0.94 \times 10^{-4}$ (ref. 11). In contrast, the frequencies of these hexamers in *Escherichia coli* DNA are $2.67 \pm 0.86 \times 10^{-4}$ and $2.17 \pm 0.87 \times 10^{-4}$, respectively (J. Han, personal communication), not significantly different from that of the non-mitogenic hexamers in humans.

Antisense oligonucleotides targeted to complementary messenger RNAs are popular research tools[12–15] and are now in human clinical trials[16–18]. It has become increasingly clear that oligonucleotides have many non-antisense effects[15,20,21]. Our review of the literature reveals 18 reports in which oligonucleotides that cause *in vitro* or *in vivo* lymphocyte activation or protection from apoptosis contain excellent matches for the CpG motif whereas control oligonucleotides do not (list available from authors on request). It may be desirable to avoid unmethylated CpGs in 'antisense' oligonucleotides, or at least to include controls for these effects. By contrast, immune-stimulating CpG oligonucleotides may prove clinically useful as adjuvants and biological response modifiers. □

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Anti-inflammatory properties of a platelet-activating factor acetylhydrolase

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PLATELET-ACTIVATING factor (PAF) is a potent pro-inflammatory phospholipid that activates cells involved in inflammation[1,2]. The biological activity of PAF depends on its structural features, namely an ether linkage at the *sn*-1 position and an acetate group at the *sn*-2 position. The actions of PAF are abolished by hydrolysis of the acetyl residue, a reaction catalysed by PAF acetylhydrolase. There are at least two forms of this enzyme—one intracellular and another that circulates in plasma and is likely to regulate inflammation. Here we report the molecular cloning and characterization of the human plasma PAF acetylhydrolase. The unique sequence contains a Gly-Xaa-Ser-Xaa-Gly motif commonly found in lipases. Recombinant PAF acetylhydrolase has the substrate specificity and lipoprotein association of the native enzyme, and blocks inflammation *in vivo*: it markedly decreases vascular leakage in pleurisy and paw oedema, suggesting that PAF acetylhydrolase might be a useful therapy for severe acute inflammation.

We purified PAF acetylhydrolase from human plasma (Fig. 1 legend). As macrophages secrete PAF acetylhydrolase activity *in vitro*[3,4], we made a plasmid complementary DNA library from messenger RNA from monocyte-derived macrophages[5], which we screened using a combination of the polymerase chain reaction (PCR) and hybridization for the unique N-terminal sequence (IQVLMAAASFGQTKIP) of the purified protein (M_r 44K). A full-length clone (sAH406-3) contains a 1.5-kilobase (kb) insert that encodes a predicted 441-amino-acid protein (Fig. 1). The open reading frame is defined by a translation initiation codon at nucleotide 160 that is preceded by a stop codon 24 base pairs (bp) upstream. The translation termination codon at the 3′ end lies immediately upstream of the predicted polyadenylation sequence (ATTAAA). Interestingly, the amino terminus determined by protein sequencing lies 42 residues downstream of the predicted initiating methionine. Hydropathy analysis suggests a hydrophobic signal peptide within the first 20 residues and, consistent with this, Ala 17/Val 18 constitutes a reasonable consensus signal cleavage position[6]. Lys 41 Ile 42 is unlikely to

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Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like receptor 9

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Abstract

Synthetic oligodeoxynucleotides (ODN) expressing non-methylated "CpG motifs" patterned after those present in bacterial DNA have characteristic immunomodulatory effects. CpG DNA is recognized as a pathogen-associated molecular pattern, and triggers a rapid innate immune response. CpG ODN are being harnessed for a variety of therapeutic uses, including as immune adjuvants, for cancer therapy, as anti-allergens, and as immunoprotective agents. The signal transduction pathway mediated by the engagement of CpG DNA with Toll-like receptor 9 (TLR9) is shared with other members of the TLR family. Recent studies demonstrate that formation and maturation of CpG DNA-containing endosomes are regulated by phosphatidylinositol 3 kinases and the Ras-associated GTP-binding protein, Rab5, which are essential for the initiation of TLR9-mediated signaling.

Keywords: CpG DNA; TLR 9; PAMP; Signal transduction

1. TLR9 is an essential element in CpG DNA-mediated cell activation

The discovery and characterization of the Toll-like receptor (TLR) family facilitated our understanding of the innate immune system. Accumulating evidence demonstrates that individual TLRs identify determinants known as pathogen-associated molecular patterns (PAMPs) that are expressed at high frequency by infectious microorganisms but rarely (if at all) by host cells [1]. For example, lipopolysaccaride (LPS) present in the surface membrane of Gram-negative bacteria engages the TLR4/MD-2 complex, while peptidoglycan (PGN) and bacterial lipoproteins (BLPs) present in the cell wall of Gram-positive bacteria engage TLR2 [2]. The interaction of a TLR with its PAMP ligand mediates an intracellular signal that results in the production of versatile chemical mediators and cell surface molecules directing innate as well as acquired immune responses [1,3,4].

All functionally characterized TLRs signal through a common pathway involving myeloid differentiation marker 88 (MyD88), IL-1R-associated kinase (IRAK), TNFR-associated factor 6 (TRAF6), TGFb-activated kinasel (TAK1), and the kinases of IkB (IKK), IkB, and NF-kB [5,6]. Thus, TLRs are regarded as pattern recognition receptors (PRR) that sense PAMPs [1]. A series of studies support the conclusion that sequence motifs present at high frequency in bacterial but not mammalian DNA also act as PAMPs. Bacterial DNA triggers cells of the innate immune system to proliferate and become functionally active [7]. This immune stimulation is mediated by unmethylated CpG motifs present at high frequency in bacterial DNA, but rare in mammalian DNA [8,9]. The likelihood that CpG motifs act as PAMPs is further supported by evidence that the immune response they induce closely resembles that stimulated by LPS and PGN, and that responsiveness to CpG DNA is completely lost in MyD88 knock out (KO) mice [10]. Definitive evidence that CpG recognition is mediated by TLR9 was provided by studies involving TLR9 KO mice [11].

Our lab demonstrated that CpG recognition in humans is also mediated by TLR9 [12]. When several TLR9 null cells (HEK293, U87MG, HepG2, and CHOK1) were transfected with an hTLR9 expression plasmid (pCI-TLR9A) driving an NF-kB-dependent luciferase reporter plasmid, CpG DNA exposure resulted in a significant increase in luciferase activity. This stimulation was abrogated if the critical CpG motif was disrupted by inversion or methylation (see Table 1). When transfected with pCI-TLR9A, HEK293 cells produced IL-8 mRNA in response to CpG ODN but not to control

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Table 1
CpG ODN activate NF-kB expression in HEK293 cells transfected with hTLR9

Treatment	ODN sequence	Stimulation index
No Rx		1.0 + 0.2
2006	TCGTCGTTTTGTCGTTTTGTCGTT	8.0 + 0.4
K3	ATCGACTCTCGAGCGTTCTC	13.2 + 0.9
K3-flip	ATGCACTCTGCAGGCTTCTC	1.3 + 0.2
K3-methyl	AT^{m}CGACTCT^{m}CGAG^{m}CGTTCTC	1.7 + 0.1
1555	GCTAGACGTTAGCGT	2.3 + 0.2
1612	GCTAGATGTTAGCGT	2.0 + 0.2
1466	TCAACGTTGA	1.1 + 0.1
LPS		0.9 + 0.1
PGN		1.3 + 0.2

HEK293 cells were co-transfected with an hTLR9 expression vector plus an NF-kB-dependent luciferase reporter vector. After 24 h incubation with 1 μM of ODN or 1 μg/ml of LPS or PGN, luciferase activity in the cells was measured. The stimulation index reflects relative luciferase activity in cells treated with each agent vs. untreated cells. Note that ODNs 2006 and K3 contain sequences optimized for recognition by hTLR9, that K3-flip and K3-methyl represent controls in which critical CpG dinucleotides were inverted or methylated; and that ODNs 1555 and 1466 contain CpG motifs optimized for recognition by murine TLR9. Critical CpG dinucleotides are underlined. ^{m}C indicates a methyl cytosine.

ODN nor LPS, suggesting that TLR9 is an essential element in CpG DNA-mediated cellular activation [12]. Among microbial products and related compounds, CpG DNA is the only ligand for TLR9.

2. Association between CpG DNA and TLR9

The subcellular localization of CpG DNA with TLR9 was examined. HEK293 cells were transfected with a TLR9 construct tagged with a hemaggulutinin epitope (HA-TLR9). These cells were incubated with synthetic CpG ODN labeled with Cy3 plus FITC-labeled anti-HA antibody. CpG ODN and TLR9 molecules were visualized as red and green fluorescence, respectively, and their subcellular localization was assessed by confocal microscopy. In the absence of CpG DNA stimulation, TLR9 molecules localized to endocytic vesicles lying just below the plasma membrane [13]. Cy3-labeled CpG ODN associated with these vesicles, and also entered the nucleus within 10 min. By 2 h after the addition of CpG ODN to TLR9 expressing cells, the size and number of CpG ODN-containing endosomes increased significantly. Moreover, these vesicles relocated from near the plasma membrane to intracellular regions. In some cases, both CpG ODN and TLR9 molecules co-localized within the same endosomes (see Fig. 1) [12]. This distribution was not observed in cells transfected with a mutant form of HA-TLR9 lacking the intracellular domain (ICD) of TLR9, suggesting that the cryptic sorting signal present in the ICD and adequate conformation of TLR9 in the presence of the ICD are required for trafficking of TLR9 to CpG ODN-containing endosomes [12].

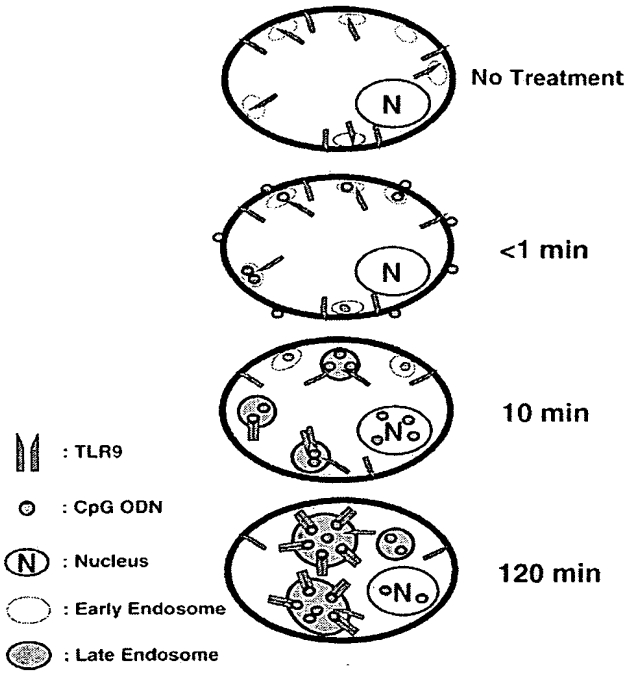

Fig. 1. Scheme of subcellular localization of Cy3-labeled CpG ODN and HA-TLR9. Under physiologic conditions, TLR9 molecules are located below the plasma membrane, presumably in vesicles. Cy3-labeled CpG ODN associate with the cell surface and are transported to these vesicles within 10 min. By 2 h the size and number of CpG ODN-containing endosomes increases significanty, and they relocate from just below the plasma membrane to intracellular regions. In some cases, the CpG ODN and TLR9 molecules co-localize within the same vesicles, recruit MyD88, and initiate the signal transduction process.

Investigators have examined the difference between TLR4- and TLR9-mediated signaling events. Results from studies involving GFP-tagged MyD88 (MyD88-GFP) in RAW264.7 macrophages indicate that the TLR4-mediated signal initiates near the plasma membrane, while the TLR9-mediated signal initiates around CpG DNA-containing endosomes [14]. These studies showed that MyD88-GFP was recruited into the lysosomal-associated membrane protein 1 (Lamp-1)-positive endosomal compartment >45 min after CpG ODN treatment.

Immune cell activation by CpG DNA and co-localization of CpG DNA with MyD88-GFP were prevented when the early steps of endocytosis were inhibited by treatment with monodansylcadaverine. The same inhibition was elicited by a dominant negative mutant of the Ras-associated GTP-binding protein, Rab5, that blocks homotypic fusion of the early endosome and endosomal trafficking [14]. These findings suggest that both formation and maturation of CpG ODN-containing endosomes are critical for the initiation of TLR9-mediated signaling.

Over evolutionary periods, TLR9 molecules expressed by different species have diverged. This has led to differ-

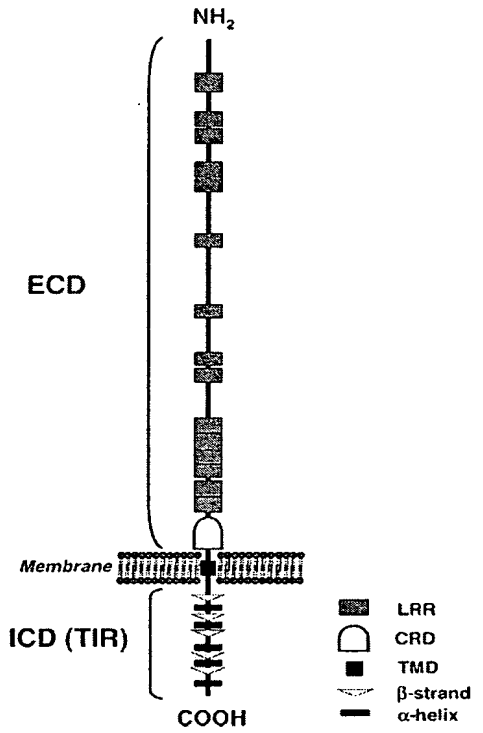

Fig. 2. Schematic representation of the molecular structure of TLR9. TLR9 is a type I membrane protein which consists of an extracellular domain (ECD), a transmembrane domain (TMD), and an intracellular domain (ICD). Leucine-rich repeats (LRRs) and a cysteine-rich domain (CRD) are present in the ECD, whereas the Toll/IL-1R homologous region (TIR) exists in the ICD.

ences in the precise sequence motif (CpG dinucleotide plus flanking regions) that optimally stimulate the innate immune system of different animals. For example, HEK293 cells transfected with murine TLR9 are more responsive to CpG motifs optimized for activity in mice (GACGTT) than humans (GTCGTT). Similarly, cells expressing human TLR9 responded optimally to GTCGTT rather than GACGTT. These findings confirm the importance of TLR9 as a receptor for CpG DNA, and suggest that this receptor accounts for species-specific differences in the response to bacterial DNA [15].

3. Cellular signaling mediated by TLR9

Computer-based analysis indicates that TLR9 is a type I membrane protein which shares common structural characteristics with other members of the TLR family. Fifteen leucine-rich repeats and one cysteine-rich domain exist within the extracellular domain (ECD) of TLR9, and a Toll/IL-1R homologous region (TIR) exists within the ICD (see Fig. 2). Studies using truncated forms of hTLR9 indicate that 32 amino acids at the COOH-terminus form a fifth a-helical structure that is critical to signaling. Co-transfection of HEK 293 cells with wild type TLR9 plus ICD deletion mutants suggest that TLR9 may function after oligomerization through the ECD interaction [12].

TLR9-mediated signaling proceeds through MyD88, an adaptor protein recruited to the TIR, which then activates the IRAK1-TRAF6-TAK1 pathway [5,6,12]. Unlike TLR4-mediated signaling, the TIR domain-containing adaptor protein/MyD88-adapter-like (TIRAP/MAL) is not involved in TLR9-mediated signaling [16,17]. Recently, a novel adapter molecule associated with MyD88-independent as well as MyD88-dependent pathways was identified [18]. Ongoing studies suggest that this molecule, TIR domain-containing adapter inducing IFN-b (TRIF), is also involved in TLR9-mediated signaling (unpublished observation).

The TLR9 signaling cascade involves mitogen-activated protein kinases (MAPKs), such as p38, c-Jun NH_2-terminal kinase (JNK), extracellular receptor kinase (ERK), and NF-kB-inducing kinase (NIK)-IKK-IkB pathways [19–21]. The activation of ERK by CpG DNA contributes to the production of IL-10 by macrophages, but is not active in dendritic cells (DCs) or B cells [20,22]. The signaling cascade culminates in the activation of several transcription factors including NF-kB, activating protein-1 (AP-1), CCAAT/enhancer binding protein (C/EBP), and cAMP-responsive element-binding protein (CREB), which directly upregulate cytokine/chemokine gene expression (see Fig. 3) [20,23–25]. In macrophages, CpG DNA also induces IFN-b production, which then upregulates STAT1 phosphorylation and IP-10 production through IFN-a/b receptor in an autocrine manner [26].

Studies using chloroquine (CQ) or wortmannin (WM) showed that these agents could block CpG DNA/TLR9 signaling but not LPS/TLR4 signaling [23,27,28]. Since cell surface binding and uptake of an ODN is not influenced by the presence of a CpG motif, endosomal maturation, which is the target of CQ, is believed to be an essential step in signaling [23,28]. Taken together with the data on the subcellular distribution of CpG DNA described above, co-localization of CpG DNA with TLR9 in endosomal vesicles, and the accompanying maturation and movement of those vesicles, seems to be involved in signaling initiation.

Although one group reported that the suppression of CpG DNA signaling by WM reflected the inhibition of DNA-dependent protein kinase (DNA-PK) [29], others find that DNA-PK KO mice and SCID mice respond normally to CpG DNA [27,30]. We observed that WM treatment led to a reduction in the size and number of endosomes containing both TLR9 and CpG ODN, suggesting that phosphatidylinositol 3 kinases (PI3K), which are also targets of WM, are involved in vesicular trafficking of CpG DNA [27]. Indeed, Rab5-mediated recruitment of class III PI3K (PI3K (III)) leads to the production of PI(3)P in the endosomal membrane, which binds to the FYVE domain of early endosome antigen 1 (EEA1), recruiting it on to the membrane. The recruited EEA1 also associates

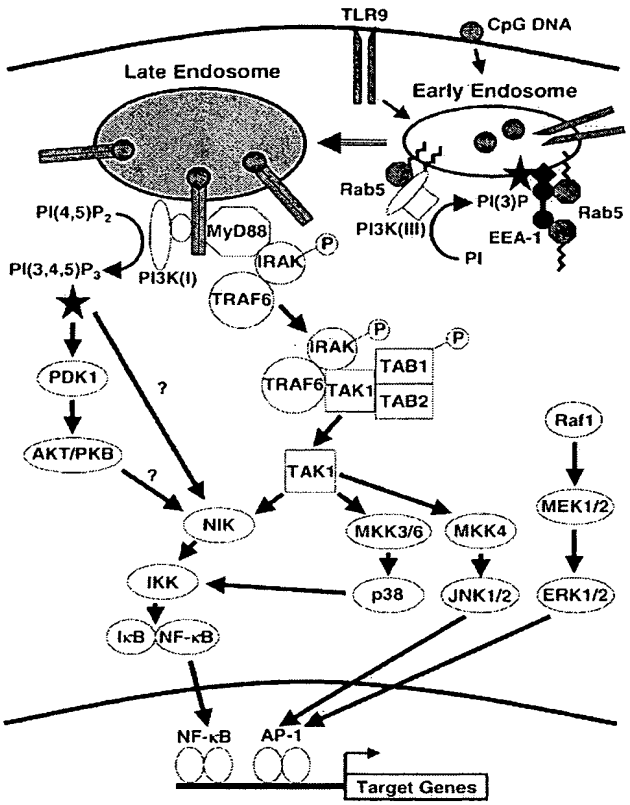

Fig. 3. Scheme of CpG DNA/TLR9-mediated cellular signaling. Class III PI3K (PI3K (III)), EEA1, and Rab5 mediate the trafficking and maturation of endosomes containing CpG DNA and TLR9, by which TLR9 transduces intracytoplasmic signal. The signal initiates with the recruitment of MyD88 to the TIR, which then activates IRAK-TRAF6-TAK1 complex. This leads to the activation of both MAPKs (JNK1/2 and P38) and IKK complex, culminating upregulation of transcription factors including NF-kB and AP-1. Raf1-MEK1/2-ERK1/2-AP-1 pathway is involved in CpG DNA-induced IL-10 production inmacrophages. The alternative pathway mediated by class I PI3K (PI3K (I))-PDK1-AKT/PKB is also suggested to be involved in TLR9-mediated cellular activation.

with Rab5 and regulates homotypic fusion and trafficking of early endosomes (see Fig. 3) [31–33]. The PI(3, 4, 5)P3, product of class I PI3K (PI3K (I)), has been demonstrated to activate a signaling cascade consisting of 3′-phosphoinositide-dependent kinase-1 (PDK1) and the protein kinase Akt/protein kinase B (AKT/PKB) [34,35]. Ligand-induced association of TLR2 ICD and PI3K (I) was reported to activate the AKT/PKB-NF-kB pathway [36]. CpG DNA also induces phosphorylation of AKT/PKB thereby inhibiting apoptosis in DCs, an effect that is reversed by a PI3K inhibitor, LY294002 [37]. However, recent data demonstrate that DN-p85a, which specifically blocks the function of PI3K (I), but neither DN-PDK1 nor DN-AKT/PKB, inhibits TLR9-mediated NF-kB activation in HEK293 cells (unpublished observation). This suggests that (1) PI3K (I) also regulate vesicular trafficking of CpG DNA and TLR9 and/or (2) another pathway mediated by PI3K(I) but not through the PDK1-AKT/PKB pathway is involved in TLR9-mediated NF-kB activation in HEK293. PI3Ks and their second messengers therefore seem to play pivotal roles at distinct steps (i.e. vesicular trafficking for the association between CpG DNA and TLR9 and the signaling pathway directing AKT/PKB activation) in CpG DNA/TLR9-mediated cellular activation.

4. Expression of TLR9 mRNA

The h*TLR9* gene consists of 2 exons, and there are two distinct spliced forms of hTLR9 mRNA. The biexonic protein, designated TLR9A, has 57 additional amino acids including a signal peptide when compared to the monoexonic protein (TLR9B) [38]. TLR9A mediates CpG DNA signaling, whereas TLR9B does not [12].

Northern blot analysis of various tissues including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis indicated that murine TLR9 (mTLR9) transcripts were most abundantly expressed in spleen [11]. Among cell types, hTLR9 mRNA is highly expressed in plasmacytoid precursor DCs ($CD11c^-$, $CD123^+$) and B cells, and to a lesser extent in monocytes [39–41]. Upon IFNg treatment, both TLR9 mRNA expression and responsiveness to CpG DNA are up-regulated in PBMC [12]. LPS also up-regulates mTLR9 mRNA expression in RAW264.7 macrophages, while colony stimulating factor-1 down-regulates constitutive mRNA expression in bone marrow-derived macrophages [42,43]. Sequence analysis of the h*TLR9* gene promoter region indicates that there are putative *cis*-elements including a TATA box, GC box, and consensus motifs for C/EBP. Transcription factors selectively expressed in a B cell linage and/or those upregulated by IFN-g or LPS may be involved in the expression of h*TLR9* gene.

5. Concluding remarks

Immune cells recognize CpG motifs using a member of the same family of TLR receptors that interact with other PAMPs, including PGN, glycolipid, and lipoprotein. Although various members of the TLR family share common structures, recent data suggest that distinct receptor–ligand associations and signaling pathways may be utilized by each member of this family. Several questions concerning the mechanism underlying CpG DNA/TLR9-mediated cellular activation currently persist. First, how do PI3Ks and their second messengers regulate trafficking and maturation of endosomes containing CpG DNA and TLR9 for signal initiation? Second, what is the precise mechanism responsible for the physical association between CpG motifs and TLR9? There is evidence that a structural change involving the TLR9 ECD and CpG motifs occurs in the acidified and Ca^{++} rich environment of the mature endosome that facil-

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itates their association. In addition, various cofactor(s) may interact with the CpG motif and regulate this association. Third, a distinct class of CpG DNA such as "D"-type ODN (also known as CpG-A ODN) induces only modest NF-kB activation in HEK293 cells expressing TLR9, but stimulates NK cells to produce a significant level of IFN-a (unpublished data). Does this class of CpG DNA stimulate NK cells through another receptor or require a distinct cofactor to utilize TLR9 for the activation? Future studies on these mechanisms should prove fruitful ground for our growing understanding of CpG DNA/TLR9-mediated immune activation.

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CpG-oligonucleotides for cancer immunotherapy : review of the literature and potential applications in malignant glioma.

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Bacterial DNA and synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODNs) are strong activators of both innate and specific immunity, driving the immune response towards the Th1 phenotype. CpG-ODNs have been successfully used in several experimental models of allergies or infections and are now entering clinical trials for these diseases. In this review, we will focus on their potential applications in cancers. CpG-ODN can be used alone to activate locally the innate immunity and trigger a tumor-specific immune response, overcoming the need for identification of a relevant tumoral antigen. Other promising approaches combined CpG-ODN with tumor antigens, monoclonal antibodies or dendritic cells. Preclinical models have shown impressive results and several clinical trials are on-going worldwide. So far, the toxicity observed in humans appeared limited, and objective responses have been observed in a few patients. In malignant gliomas, intra-tumoral injections of CpG-ODN represent a practical approach. Indeed, human gliomas display a locally invasive pattern of growth and rarely metastasize, making local treatment clinically relevant.

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Phase I study of oligodeoxynucleotide CpG 7909 in patients with previously treated non-Hodgkin's lymphoma

Sub-category: Other Novel Agents
Category: Developmental Therapeutics - Molecular Therapeutics
Meeting: 2003 ASCO Annual Meeting

Printer Friendly
E-Mail Article

Abstract No: 843

Author(s): J. Wooldridge, B. K. Link, D. J. Weisdorf, Z. K. Ballas, G. Albert, W. L. Rasmussen, A. M. Krieg, G. J. Weiner; Univ of Iowa Hosp and Clinics, Iowa City, IA; University of Minnesota, Minneapolis, MN; Coley Pharmaceutical Group, Wellesley, MA

Abstract: Background: Synthetic oligodeoxynucleotides containing CpG motifs (CpG ODN) can activate NK cells and monocytes and enhance ADCC. We report a phase I trial of CpG ODN 7909 (Promune) as a single agent with the objective of establishing a safe dose with biological activity. Methods: Pts. with previously treated NHL received 3 weekly infusions of CpG ODN 7909 (Promune) at one of 7 dose levels (DL) (3 pts each): 0.01 (DL1); 0.04 (DL2); 0.08 (DL3); 0.16 (DL4); 0.32 (DL5); and 0.64mg/kg (DL6), and 5 pts. at 0.48 mg/kg (DL7). All pts received CpG ODN 7909 IV over 2-hrs without premedication. Pts. were monitored for four weeks after the last infusion for toxicity, changes in peripheral blood effector cell function, and tumor response. Results: 23 pts. completed therapy and all but one received all scheduled infusions. 21 pts. were evaluable for effector cell changes and all patients for safety and response. The regimen was well-tolerated with infrequent transient grade 1 and 2 adverse events, including hyperglycemia, nausea, chills/rigors, hypotension, and fever. Six pts. had transient lymphopenia. Significant adverse events observed more than once included anemia (2=Gr3 2=Gr4), thrombocytopenia (4=Gr3), neutropenia (2=Gr3), and dyspnea (2=Gr2) and were largely judged due to progressive disease. Immunologic observations included: 1) Dose-related increase in ADCC mediated by PBMCs at days 7-21 over baseline (DL4 pre-therapy median = 2.33 Lytic Units, Day 21 median = 13.39 LU); 2) dose-related increased NK cell activity at day 21 over baseline observed at dose levels 1-4 (DL 4 pre-therapy median = 27.75 LU, Day 21 median = 93.95 LU). In general, immunologic activity was greater in DL 1-4 than DL 5-7. No clinical responses were documented at day 42. Partial radiographic response was observed in 2 pts at 3 months without further NHL therapy.

Conclusions: CpG ODN 7909 can be given safely to NHL pts over a variety of DL, with evidence for immunologic activity primarily within the range of 0.04-0.16 mg/kg. Phase I/II trials of CpGODN 7909 and rituximab or trastuzumab are underway.

Associated Presentation(s):

1. Phase I study of oligodeoxynucleotide CpG 7909 in patients with previously treated non-Hodgkin's lymphoma

No presentation available
Meeting: 2003 ASCO Annual Meeting
Presenter: James Wooldridge, MD
Session: Developmental Therapeutics - Molecular (General Poster Session)

Other Abstracts in this Sub-Category

1. Phase I trial of 17-AAG (17-allylamino-17-demethoxygeldanamycin) in patients (pts) with advanced cancer.

Meeting: 2003 ASCO Annual Meeting Abstract No: 795 First Author: D. B. Solit
Category: Developmental Therapeutics - Molecular Therapeutics - Other Novel Agents

2. A pharmacokinetically (PK) - pharmacodynamically (PD) guided phase I trial of the heat shock protein 90 (HSP90) inhibitor 17-allylamino,17-demethoxygeldanamycin (17AAG)

Meeting: 2003 ASCO Annual Meeting Abstract No: 797 First Author: U. Banerji
Category: Developmental Therapeutics - Molecular Therapeutics - Other Novel Agents

3. A phase I study to determine the safety and pharmacokinetics (PK) of BMS-387032 given intravenously every three weeks in patients with metastatic refractory solid tumors.

Meeting: 2003 ASCO Annual Meeting Abstract No: 798 First Author: S. F. Jones
Category: Developmental Therapeutics - Molecular Therapeutics - Other Novel Agents

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Abstracts by J. Wooldridge

1. Improved survival of follicular lymphoma patients in the surveillance, epidemiology, and end-results (SEER) program.

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Category: Adult Leukemia, Lymphoma and Transplantation - Lymphoma

2. Integrated PET based response classification for non-Hodgkin's lymphoma (NHL).

Meeting: 2004 ASCO Annual Meeting Abstract No: 6533 First Author: M. E. Juweid
Category: Adult Leukemia, Lymphoma and Transplantation - Lymphoma

3. Phase I study of oligodeoxynucleotide CpG 7909 in patients with previously treated non-Hodgkin's lymphoma

Meeting: 2003 ASCO Annual Meeting Abstract No: 843 First Author: J. Wooldridge
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Presentations by J. Wooldridge

1. Phase I study of oligodeoxynucleotide CpG 7909 in patients with previously treated non-Hodgkin's lymphoma

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J Clin Oncol, United States
Vol 23, No 22 (6/29/2005): pp. 5019-26
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2. Response assessment of aggressive non-Hodgkin's lymphoma by integrated international workshop criteria and fluorine-18-fluorodeoxyglucose positron emission tomography.

J Clin Oncol, United States
Vol 23, No 21 (4/20/2005): pp. 4652-61
PMID: 15837965 [PubMed - in process]

3. Serum alters the uptake and biologic activity of CpG oligodeoxynucleotides in B cell chronic lymphocytic leukemia.

Oligonucleotides, United States
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Phase Ib trial of a targeted TLR9 CpG immunomodulator (CPG 7909) in advanced renal cell carcinoma (RCC)

Sub-category: Kidney Cancer
Category: Genitourinary Cancer
Meeting: 2004 ASCO Annual Meeting

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E-Mail Article

Abstract No: 4644

Author(s): J. A. Thompson, T. Kuzel, R. Bukowski, F. Masciari, T. Schmalbach; Seattle Cancer Care Alliance, Seattle, WA; Northwestern University, Chicago, IL; Cleveland Clinic Foundation, Cleveland, OH; Coley Pharmaceutical Group, Wellesley, MA

Abstract: Background: Patients with metastatic RCC have a poor prognosis, with infrequent responses to immunotherapy. CPG 7909, one of a new class of oligodeoxynucleotide immunomodulators, binds Toll-like receptor 9 on dendritic cells, with induction of cytokines including IFNÎ±, activation of NK cells, and modulation of a Th1 CTL environment. Early trials of CPG 7909 demonstrated anti-tumor activity in human cancers at well-tolerated doses. Based upon the medical need and the novel mechanism of action, the current trial evaluates CPG 7909 in the treatment of metastatic RCC. Methods: This multi-center, dose escalation trial evaluates weekly s.c. doses of CPG 7909 with cohorts of patients given 0.08, 0.12, 0.16, 0.36, 0.54 or 0.81 mg/kg for 24 weeks or until progression. Patients had prior nephrectomy but no systemic therapy for clear-cell RCC, ECOG performance â‰¤1, with measurable soft tissue metastases (bone, liver, brain excluded). The primary endpoint is safety, with secondary endpoints including pharmacokinetic and pharmacodynamic parameters, responses by RECIST, duration of response, time to progression, and survival. Results: Thirty-one patients have enrolled, 18 males and 13 females, ages 35-79. Twenty-seven patients are off-study prior to, or at 24 weeks of treatment. One patient had a durable PR (8 months), 9 had stable disease and 17 patients progressed on CPG 7909. Four patients continue on therapy. Median time to progression is 112 days. No drug-related serious adverse events have been reported, with good tolerability up to 0.54 mg/kg. Pro-inflammatory or cytokine effects (erythematous injection-site reactions, chills, myalgias, arthralgias and fatigue) are dose related and reversible. Biologic responses are consistent with the CpG TLR9 mechanism of action; the most consistent effects are increased plasma IP-10 and 2â€™5 OAS.

Conclusions: In this ongoing dose-escalation study, CPG 7909 can be safely administered at doses up to 0.54 mg/kg weekly. Correlations of PK, PD, clinical symptoms and response will be provided.

Associated Presentation(s):

1. **Phase Ib trial of a targeted TLR9 CpG immunomodulator (CPG 7909) in advanced renal cell carcinoma (RCC)**

   Meeting: 2004 ASCO Annual Meeting
   Presenter: John A. Thompson, MD
   Session: Genitourinary Cancer (General Poster Session)
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1. **SU011248, a novel tyrosine kinase inhibitor, shows antitumor activity in second-line therapy for patients with metastatic renal cell carcinoma: Results of a phase 2 trial.**

   Meeting: 2004 ASCO Annual Meeting Abstract No: 4500 First Author: R. J. Motzer
   Category: Genitourinary Cancer - Kidney Cancer
2. **Phase II trial of bevacizumab and erlotinib in patients with metastatic renal carcinoma (RCC).**

   Meeting: 2004 ASCO Annual Meeting Abstract No: 4502 First Author: J. D. Hainsworth
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3. **Carbonic Anhydrase IX (CAIX) expression predicts for renal cell cancer (RCC) patient response and survival to IL-2 therapy.**

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1. **Preliminary tolerability and anti-tumor activity of intravenous recombinant human Interleukin-21 (IL-21) in patients with metastatic melanoma and metastatic renal cell carcinoma**

   Meeting: 2005 ASCO Annual Meeting Abstract No: 2502 First Author: B. D. Curti
   Category: Developmental Therapeutics: Immunotherapy - Cytokines

2. **Phase Ib trial of a targeted TLR9 CpG immunomodulator (CPG 7909) in advanced renal cell carcinoma (RCC)**

   Meeting: 2004 ASCO Annual Meeting Abstract No: 4644 First Author: J. A. Thompson
   Category: Genitourinary Cancer - Kidney Cancer

3. **Phase Î™Î™ trial evaluating the safety and efficacy of EPO906 in patients with advanced renal cancer.**

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2. **Are We Making Progress in Advanced Renal Cell Cancer?**

   Meeting: 2004 ASCO Annual Meeting
   Discussant: John A. Thompson, MD
   Session: Genitourinary (Nonprostate) Cancer (Oral Presentation)
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3. **Phase Ib trial of a targeted TLR9 CpG immunomodulator (CPG 7909) in advanced renal cell carcinoma (RCC)**

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1. **The absolute bioavailability of oral vinorelbine in patients with solid tumors.**

Cancer Chemother Pharmacol, Germany
Vol 56, No 6 (7/8/2005): pp. 578-84
PMID: 16001165 [PubMed - in process]

2. **A phase II open-label trial of apomine (SR-45023A) in patients with refractory melanoma.**

Invest New Drugs,
Vol , No (12/28/2005): pp.
PMID: 16379038 [PubMed - in process]

3. **Fibroblast growth factor-1 induces heme oxygenase-1 via nuclear factor erythroid 2-related factor 2 (Nrf2) in spinal cord astrocytes: consequences for motor neuron survival.**

J Biol Chem, United States
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TLR9-targeted CpG immunostimulatory treatment of metastatic melanoma: A phase II trial with CpG 7909 (ProMune).

Sub-category: Melanoma
Category: Melanoma
Meeting: 2004 ASCO Annual Meeting

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E-Mail Article

Abstract No: 7513

Author(s): S. N. Wagner, M. Pashenkov, G. Goess, C. Wagner, A. Schneeberger, A. M. Krieg, G. Stingl, C003 Study Group; Medical University, Vienna, Austria; Coley Pharmaceutical Group, Inc., Wellesley, MA

Abstract: Background: Malignant melanoma is widely accepted as an immunologically responsive tumor, but large recently reported chemobiotherapy trials have been disappointing and prognosis is poor. The new synthetic oligodeoxynucleotide CpG 7909 activates plasmacytoid dendritic cells (pDC) and B cells through specific interaction with Toll-like receptor 9 (TLR9) and is a strong activator of both innate and specific immunity. CPG 7909 crossreacts with mouse TLR9 and has shown impressive antitumor activity in preclinical tumor models when used as monotherapy. Methods: Patients with confirmed metastatic melanoma (stage IV without CNS metastases) were enrolled into an open-label, multicenter, single arm study. Pts received 6 mg CPG 7909 weekly by SC injection for 24 weeks or until disease progression in an outpatient setting. Disease status was assessed at screening and weeks 8, 16, and 24 according to RECIST. Results: 20 pts (5 female, 15 male, aged between 37 and 74) were enrolled. Two pts achieved a confirmed partial response (PR) and one has maintained this response with continuing therapy for over 13 months. Three pts achieved stable disease (SD). CPG 7909 was well tolerated. Adverse events included transient injection site reactions (erythema, swelling, induration), fever and arthralgias. Hematological and non-hematological toxicities were limited, transient and did not result in any withdrawals.. Phenotyping of PBMC revealed activation of pDC under CPG 7909 therapy consistent with the mode of action and pts exhibiting PR or SD could be distinguished from non-R pts by differential dynamics in NK cell-cytotoxicity (1.6-fold increase vs. 1.7-fold decrease in lytic units, $p<0.05$) during the first 8 weeks of treatment. Conclusions: CPG 7909 exerts anti-tumor activity in pts with metastatic melanoma, can be administered safely and induces a phenotypic signature in PBMC associated with exposure and, possibly, response to therapy. A randomised phase II/III trial has been

initiated to compare efficacy and safety of two dose levels of CPG 7909, CPG 7909 in combination with DTIC, and DTIC alone.

Associated Presentation(s):

1. **TLR9-targeted CpG immunostimulatory treatment of metastatic melanoma: A phase II trial with CpG 7909 (ProMune).**

   Meeting: 2004 ASCO Annual Meeting
   Presenter: Stephan N. Wagner, MD
   Session: Melanoma (Poster Discussion)
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Other Abstracts in this Sub-Category

1. **A population-based validation of the AJCC melanoma staging system.**

   Meeting: 2004 ASCO Annual Meeting Abstract No: 7500 First Author: D. Guerry
   Category: Melanoma
2. **Significant impact of HLA class I allele expression on outcome in melanoma patients treated with an allogeneic melanoma cell lysate vaccine. Final analysis of SWOG-9035.**

   Meeting: 2004 ASCO Annual Meeting Abstract No: 7501 First Author: V. K. Sondak
   Category: Melanoma
3. **E1696: Final analysis of the clinical and immunological results of a multicenter ECOG phase II trial of multi-epitope peptide vaccination for stage IV melanoma with MART-1 (27-35), gp100 (209-217, 210M), and tyrosinase (368-376, 370D) (MGT) +/â^' IFNÎ±2b and GM-CSF.**

   Meeting: 2004 ASCO Annual Meeting Abstract No: 7502 First Author: J. M. Kirkwood
   Category: Melanoma

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Abstracts by S. N. Wagner

1. **TLR9-targeted CpG immunostimulatory treatment of metastatic melanoma: A phase II trial with CpG 7909 (ProMune).**

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1. **TLR9-targeted CpG immunostimulatory treatment of metastatic melanoma: A phase II trial with CpG 7909 (ProMune).**

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CPG 7909, a TLR9 Agonist, Added to First Line Taxane/Platinum for Advanced Non-Small Cell Lung Cancer, A Randomized, Controlled Phase II Study

Sub-category: Non-Small Cell Lung Cancer
Category: Lung Cancer
Meeting: 2005 ASCO Annual Meeting

Abstract No: 7039

Author(s): G. Leichman, D. Gravenor, D. Woytowitz, J. Mezger, G. Albert, T. Schmalbach, M. Al-Adhami, C. Manegold

Abstract: Background: First line treatment for stage IIIb/IV NSCLC continues to be a combination of a taxane + platinum with partial responses expected in only 20 - 30 % of patients. Multiple pre-clinical models have shown that tumor response and survival after chemotherapies are significantly improved by the addition of CPG 7909. This new synthetic oligodeoxynucleotide acts through dendritic cells by targeting the Toll-like Receptor 9 with subsequent immunomodulatory effects. Human dose ranging, safety and biologic responses to weekly S.C. doses of CPG 7909 have been established in earlier trials. Methods: This phase II trial randomized and treated 112 patients in 23 centers. NaÃ¯ve patients with NSCLC were randomized 2:1 and received 4 - 6 three-week cycles of a standard regimen of taxane and platinum plus CPG 7909 [C + CPG 7909] on weeks 2 and 3 of each cycle or [C] alone. Each dose of CPG 7909 was 0.20 mg/kg given S.C. The primary endpoint of objective response was assessed at cycles 2, 4 and 6 using RECIST criteria. A *post-hoc* independent radiological review [IRR] was conducted on coded and blinded scans. Secondary efficacy analyses are being completed, and include clinical benefit, time to and duration of response, and survival. Biomarker responses to CPG 7909 will be compared for responders vs non-responders in the two arms. Results: Data for an ITT response analysis are available on 83 patients from the planned protocol (57 vs 26). Baseline demographics are balanced except for more stage IV patients in the C + CPG 7909 group. Administration of CPG 7909 did not complicate standard cycles of [C]. ORR was 23 % vs 40 % for the [C] vs [C+CPG 7909] arms, and is supported by the retrospective IRR data. Kaplan-Meier curves show a trend to improved PFS and survival in [C + CPG 7909] compared to [C]. A complete analysis including follow up survival will be presented on the 83 planned patients plus 28 patients more recently enrolled in a protocol amendment. Conclusions: A well tolerated regimen of weekly CPG

7909 added to a taxane/platinum regimen for first line treatment of NSCLC appears to improve objective response, and pivotal studies are warranted to document clinical benefit of this new modality.

Associated Presentation(s):

1. CPG 7909, a TLR9 agonist, added to first line taxane/platinum for advanced non-small cell lung cancer, a randomized, controlled phase II study

   Meeting: 2005 ASCO Annual Meeting
   Presenter: Gail Leichman, MD
   Session: Lung Cancer I (Poster Discussion)
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   Category: Lung Cancer - Non-Small Cell Lung Cancer
2. Paclitaxel and gemcitabine vs carboplatin and gemcitabine. A multicenter, phase III randomized trial in patients with advanced inoperable Non-small cell lung cancer (NSCLC).

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Abstracts by G. Leichman

1. CPG 7909, a TLR9 Agonist, Added to First Line Taxane/Platinum for Advanced Non-Small Cell

Lung Cancer, A Randomized, Controlled Phase II Study

Meeting: 2005 ASCO Annual Meeting Abstract No: 7039 First Author: G. Leichman
Category: Lung Cancer - Non-Small Cell Lung Cancer

2. A TLR9 CpG immunomodulator in combination with chemotherapy as treatment for advanced non-small cell lung cancer (NSCLS), a randomized, controlled phase II study.

Meeting: 2004 ASCO Annual Meeting Abstract No: 7126 First Author: G. Leichman
Category: Lung Cancer - Non-Small Cell Lung Cancer

3. Updated results of an exploratory gene expression analysis for primary esophageal cancer (PEC) patients (pts) treated with oxaliplatin (OXP), protracted infusion (PI) 5FU and radiation (XRT).

Meeting: 2004 ASCO Annual Meeting Abstract No: 4014 First Author: L. P. Leichman
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Presentations by G. Leichman

1. CPG 7909, a TLR9 agonist, added to first line taxane/platinum for advanced non-small cell lung cancer, a randomized, controlled phase II study

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Session: Lung Cancer (General Poster Session)

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Carpentier, A., Laigle-Donadey, F., Zohar, S., Capelle, L., Behin, A., Tibi, A., Martin-Duverneuil, N., Sanson, M., Lacomblez, L., Taillibert, S., Puybasset, L., Van Effenterre, R., Delattre, J.-Y., Carpentier, A.F. Phase 1 trial of CpG ODN for patients with recurrent glioblastoma. *Neuro-Oncology* [serial online], Doc. 05-047, November 29, 2005. URL http://neuro-oncology.mc.duke.edu; DOI: 10.1215/S1552851705000475

Phase 1 trial of a CpG oligodeoxynucleotide for patients with recurrent glioblastoma[1]

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Oligodeoxynucleotides containing CpG motifs (CpG ODNs) display a strong immunostimulating activity and drive the immune response toward the Th1 (T helper type 1) phenotype. These ODNs have shown promising efficacy in preclinical studies when injected locally in several cancer models. We conducted a phase 1 trial to define the safety profile of CpG-28, a phosphorothioate CpG ODN, administered intratumorally by convection-enhanced delivery in patients with recurrent glioblastoma. Cohorts of three to six patients were treated with escalating doses of CpG-28 (0.5–20 mg), and patients were observed for at least four months. Twenty-four patients entered the trial. All patients had previously been treated with radiotherapy, and most patients had received one or several types of chemotherapy. Median age was 58 years (range, 25–73) and median KPS was 80% (range, 60%–100%). Adverse effects possibly or probably related to the studied drug were moderate and consisted mainly in worsening of neurological conditions (four patients), fever above 38°C that disappeared within a few days (five patients), and reversible grade 3 lymphopenia (seven patients). Only one patient experienced a dose-limiting toxicity. Preliminary evidence of activity was suggested by a minor response observed in two patients and an overall median survival of 7.2 months. In conclusion, CpG-28 was well tolerated at doses up to 20 mg per injection in patients with recurrent glioblastoma. Main side effects were limited to transient worsening of neurological condition and fever.

Keywords: convection-enhanced delivery, CpG ODN, glioblastoma, glioma, immunotherapy, oligodeoxynucleotide, phase 1

Glioblastoma is the most frequent malignant glioma in adults. Despite surgical resection and radiotherapy, the prognosis in these patients remains poor, with a median survival of around 12 months. When glioblastoma recurs, the efficacy of chemotherapy is very limited and the median survival is around six months (Brada et al., 2001; Yung et al., 2000).

Synthetic phosphorothioate oligodeoxynucleotides (ODNs) containing unmethylated CpG dinucleotides

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Dr. Antoine Carpentier holds a patent position on cancer immunotherapy with CpG ODNs. The Assistance Publique—Hôpitaux de Paris is co-owner of a patent on CpG.

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[3]Abbreviations used are as follows: DLT, dose-limiting toxicity; MTD, maximum tolerated dose; ODN, oligodeoxynucleotide; SS, Sjögren's syndrome; Th1, T helper type 1.

(CpG ODNs) are strong activators of both innate and adaptive immunity. They drive the immune response toward the Th1 (T helper type 1) phenotype, which promotes CD8+ cellular cytotoxicity (Klinman, 2004; Krieg, 2004). Given their potent Th1 adjuvant properties, CpG ODNs have been introduced into clinical trials in vaccine protocols combined with the hepatitis B surface antigen or with Fluarix influenza vaccine (GlaxoSmithKline Biologicals, Rixensart, Belgium) (Cooper et al., 2004a, b; Halperin et al., 2003). In cancer research, the identification of tumor antigens is a limiting step for the design of therapeutic vaccines. To overcome this problem, CpG ODNs alone can be directly injected into the tumor, with the expectation that the immune system will select the most relevant antigens. In addition, CpG ODNs activate innate immunity (natural killer cells and macrophages), which can directly kill tumor cells. The validity of such an approach was shown in a neuroblastoma model, in which peritumoral injections of a synthetic ODN containing a CpG motif induced complete tumor rejection in the majority of mice and triggered a long-term immunity (Carpentier et al., 1999). Further studies have confirmed the antitumor effects of CpG ODNs in various cancer models, including malignant glioma (Carpentier et al., 2003). In an intracranial model of syngeneic glioma (CNS1), more than 85% of the rats treated five days after tumor inoculation with a single intratumoral injection of CpG ODNs showed long-term survival and tumor eradication. Rats that were cured by CpG ODN injections were further protected against new tumor challenge, showing that a long-term immunity was primed (Carpentier et al., 2000).

On the basis of our preclinical data, we initiated a phase 1 trial to assess the feasibility and safety of local injections of CpG ODNs in patients with recurrent glioblastoma. CpG-28, the CpG ODN that gave the best results in preclinical glioma models (Meng et al., 2005), was selected for this trial. Direct infusion of CpG-28 into brain tumors was achieved by implanted catheters and high-flow microinfusion. This technique allows fluids to be distributed by bulk flow (convection) through the interstitial spaces and spread throughout distant areas of the brain (Broaddus et al., 1998).

Patients and Methods

Patient Eligibility Criteria

Eligibility criteria were defined as follows: histologically proven glioblastoma, disease progression documented at least three months after surgery and radiotherapy, measurable contrast enhancement (>1 cm) on MRI, KPS score of 50 or higher, four-week interval from last chemotherapy (six-week interval for nitrosourea), and adequate bone marrow (platelet count >100,000) and hepatic function (plasma aspartate aminotransferase and alanine aminotransferase levels <3 times the normal limit). Ineligibility criteria were pregnancy, past history of autoimmune disease or multiple sclerosis, and recurrent tumor considered resectable by the neurosurgeon.

All patients signed an informed consent form before being included in the study, which was approved by the institutional review board. Concurrent systemic chemotherapy was not allowed until tumor progression.

Treatment Design and Dose Escalation

CpG-28 (sequence 5'-TAAACGTTATAACGTTATGACGTCAT-3') (Carpentier et al., 2003; Meng et al., 2005) was synthesized with a wholly phosphorothioate backbone by Avecia (Milford, U.K.) and was supplied by the Agence Générale des Equipements et Produits de Santé (Paris, France) in vials containing 10 mg of CpG-28. When needed, the drug was further diluted in 0.9% sodium chloride, so that the total drug volume injected was 1 ml per implanted catheter. Catheters were placed by using stereotactic guidance through small twist-drill holes with patients under local anesthesia. No surgical resections of the tumors were performed. The tips of the catheters targeted the contrast-enhanced areas, and they had to be at least 2 cm deep within the brain. After surgery, a CT scan was performed to verify the position of the catheters and the absence of any hemorrhage, and the externalized catheters were connected to a 5-ml syringe filled with CpG-28. The infusion began 1 h after implantation at an infusion rate of 0.2 ml/h (DPS-orchestra electric syringe; Fresenius Vial, France) for 6 h (1 h for the dead volume of the catheter and 5 h for drug infusion). In addition to their usual treatment, patients were premedicated with 1 mg diazepam to prevent seizures. The catheters were removed 1 h after completion of drug administration.

The dose of CpG-28 was escalated from 0.5 mg (level 1) to 1.0 mg (level 2) to 2.0 mg (level 3) to 5.0 mg (level 4) to 10.0 mg (level 5) to 20.0 mg (level 6). One implanted catheter was used for levels 1 to 4 and for the first three patients of level 5. The protocol was then amended to infuse CpG-28 through two catheters instead of one. Three additional patients were then treated at the same dose (level 5) before the dose was escalated to level 6.

Follow-up

Patients were assessed two days, 15 days, and monthly for four months after injection. At each follow-up visit, a neurological and a general examination, a KPS rating, and an MRI with contrast media were performed (except on day 2, when a CT scan was allowed). Complete blood count, serum biochemistry, and liver function tests were repeated at each visit. Antinuclear antibody and anti-SSA/SSB (Sjögren's syndrome) antibody titers were measured at baseline and day 30. Toxicity was graded according to the NCI expanded Common Toxicity Criteria (NCI CTC 2.0). Radiographic response was evaluated by using the Macdonald criteria (Macdonald et al., 1990). MRI images were centrally reviewed by the same radiologist (N.M.-D.).

Patients were allowed to receive chemotherapy if evidence for tumor progression was seen after day 60. Even in those cases, the patients were monitored for four months after drug administration.

Study Design and Quality Insurance

This phase 1 trial was designed as an open-label, nonrandomized study, in which groups of three to six patients were treated with escalating doses of CpG-28. The first three patients at a dose level were observed for four weeks after drug administration. If no dose-limiting toxicity (DLT) was observed among those three patients, the dose would be escalated to the next level. If one instance of DLT was observed among the initial three patients treated at a dose level, an additional three patients had to be treated at that dose level with no further DLT for dose escalation to proceed. If two instances of DLT were observed at a dose level, the maximum tolerated dose (MTD) was surpassed, and a total of six patients had to be treated at the previous level to ensure its tolerability. The MTD was the highest dose to cause DLT in no more than one of six patients at that dose level.

DLT was defined as severe peritumoral edema resistant to steroids and leading to intracranial hypertension; neurological deterioration with a Rankin score decrease of more than one point that was unrelated to tumor progression and that lasted more than 15 days; or grade 4 nonhematopoietic or hematologic toxicity occurring within one month after administration of CpG-28. Evidence for severe autoimmune diseases or multiple sclerosis was also considered to be DLT, irrespective of the delay after drug administration.

All recorded data were monitored by an independent scientific committee that approved each dose escalation. (This scientific committee was composed of one neurosurgeon [H. Loiseau], one neuro-oncologist [O. Chinot], one neuroradiologist [F. Lafitte], one oncologist [E. Raymond], and one statistician [B. Asselain]).

Statistical Analysis

Demographic and baseline characteristics were recorded as medians (with ranges) for continuous variables and proportion for categorical variables. All adverse events occurring within the first 30 days after drug administration were analyzed by dose group. A Kaplan–Meier method was used to estimate overall survival after study enrollment.

Results

Patient Characteristics

Twenty-four patients were enrolled in this single-center trial between January 2003 and June 2004. Nine patients (37%) were included at the time of first recurrence, eight (33%) at the time of second recurrence, and seven (29%) at the time of third recurrence. Most patients were treated with steroids at the time of study enrollment (prednisone equivalent dose: >60 mg/day, two patients; 30–60 mg/day, nine patients; 5–30 mg/day, 10 patients; and no steroids, three patients). The clinical characteristics of patients are outlined in Table 1.

Dose Escalation and Dose-Limiting Toxicity

One adverse event was considered to be a probable DLT (patient 21 at a 20-mg dose). This patient was rapidly deteriorating at the time of inclusion, experienced a transient worsening after drug injection (increased aphasia, hemiparesis, and visual disorders), and did not fully recover despite increased steroid levels. Although it was unclear whether this incomplete recovery was related to tumor progression or injection of CpG-28, these symptoms were considered a DLT. Consequently, three additional patients were treated at this dose level, but no further DLTs were observed.

Safety and Tolerability

There were 120 adverse events reported, and 63 were considered as possibly related to the investigated treatment (Table 2). Adverse events (≥grade 2) possibly or probably related to CpG-28, according to the investigator in charge of the patient, are summarized in Table 3.

Table 1. Characteristics of patients treated with CpG ODN

Characteristic	No. of Patients*	Percent
Sex		
Male	14	58
Female	10	42
Age, years		
Median	58	
Range	25–73	
KPS		
Median	80%	
Range	60–100%	
100%	2	8
90%	5	21
80%	6	25
70%	6	25
60%	5	21
Tumor histology, glioblastoma	24	100
Time from first diagnosis to treatment, months		
Median	11	
Range	6–62	
Prior therapy		
Surgery**	23	96
No. of interventions before enrollment		
1	18	75
2	5	21
Radiotherapy	24	100
Chemotherapy	21	88
No. of regimens before enrollment		
1	10	42
2	6	25
3	5	21

Abbreviations: KPS, Karnofsky performance status; ODN, oligodeoxynucleotide.

*Medians, ranges, and percentages as indicated.

**Except biopsies.

Table 2. Reported adverse events (grade >2)

Adverse Event	Probably Related	Possibly Related	Probably Unrelated
Clinical AEs			
Partial seizures	0	5	7
General seizures	0	0	2
Fever (grade 2)	0	4	0
Fever (grade 3)	0	1	0
Worsening of previous neurological condition	1	4	15
Somnolence (grade ≥2)	0	2	4
Nausea (grade 2)	0	1	3
Fatigue (grade 2)	1	5	0
Other clinical symptoms (grade ≥2)	0	0	9
Biological AEs*			
Lymphopenia (grade 2)	0	20	6
Lymphopenia (grade 3)	0	9	1
ALT (grade ≥2	0	6	1
AST (grade 2)	0	1	0
Other biological alterations (grade ≥2)	0	3	9

Abbreviations: AE, adverse event; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

*Counted several times if they lasted over several follow-up visits.

Except for the patient with DLT, the adverse events were generally mild and well tolerated, the most frequent being lymphopenia.

Transient worsening of baseline neurological symptoms was seen in one patient on dose 4 and in three patients on level 6. In patient 21, the worsening was considered as a DLT (see above). In patients 11, 22, and 23, this worsening was moderate and resolved within two weeks. Patient 4 experienced nausea and somnolence four days after injection, but the relationship with CpG-28 is unclear because the patient had similar symptoms before inclusion. When a neurological worsening did occur, although no increase in peritumoral edema was seen on MRI, steroid dosage was increased. In addition, five patients complained of grade 2 fatigue for a few days after injection (two patients on level 5 and three patients on level 6).

Increased body temperature (>38°C) was noted in five patients (one patient in level 3, two patients in level 5, and two patients in level 6). Fever peaked on day 3 (maximum of 39.3°C in one patient), was well tolerated, and disappeared within five days without antibiotics.

In the month following administration of CpG-28, five patients had short partial seizures. In patient 19, a relationship with the treatment is possible because the seizure occurred just after administration of CpG-28. The other patients had occasional seizures before enrollment, and the relationship with the studied drug is unclear.

Two adverse events were considered as probably related to the procedure. Patient 1 was on long-term anticoagulant therapy for a past history of pulmonary embolism diagnosed six years before inclusion. This treatment was discontinued by the time of enrollment into this trial. A pulmonary embolism and right-leg deep-vein thrombosis were diagnosed 37 days after administration of CpG-28. The anticoagulation treatment was then resumed, and the patient slowly recovered. Patient 11 experienced neurological worsening and a partial seizure on day 5 after drug administration. MRI on day 15 showed a T1 hypersignal suggesting a small hemorrhage at the site of catheter implantation. Although the exact date of onset cannot be determined, it is likely that this hemorrhage was related to catheter implantation. The patient recovered within two weeks.

Seven patients experienced grade 3 lymphopenia ($<500/mm^3$), three of whom already had a grade 2 lymphopenia at the time of inclusion. All these patients had chemotherapy between one and four months before study enrollment. The decrease in lymphocyte counts on day 30 (when compared to the day of preinclusion screening) was not statistically significant (mean ± SD, 982 ± 596 vs. 1367 ± 1191/mm^3; $P = 0.38$). This lymphopenia resolved in all cases by day 60 and was not associated with any infectious diseases. The relationship with the studied drug is possible, although no dose-response relationship was found.

Grade 3 nonhematological toxicities were limited to reversible alanine aminotransferase elevation in two patients and hyponatremia in one patient treated at the highest dose. These biochemical alterations were reversible, and their relationship with the studied drug is unclear. A moderate elevation of antinuclear antibody titers at day 30 was seen in patient 4 (nondetectable to 1:320) and patient 18 (1:160 to 1:320) without symptoms associated with autoimmune disease. Anti-SSA/SSB antibodies were undetectable in all patients.

There were no treatment-related deaths, and we found no evidence for drug-induced edema, increased mass effect, or autoimmune disease after administration of CpG-28.

Table 3. Adverse events (grade ≥2) possibly or probably related to administration of CpG-28

Patient	Dose (mg)	Fever >38°C	Clinical Symptoms Within 30 Days	Lymphopenia	Liver Toxicity	Other Events
1	0.5			Grade 3, day 30	Grade 2, day 15	Pulmonary embolism, day 37
2			Nausea, somnolence on day 4	Grade 3, day 30		
3						
4	1			Grade 3, day 30		
5						
6						
7	2			Grade 2, day 30		
8						
9				Grade 3, day 15		
10	5					
11		38.3, day 1	Transient neurological worsening Partial seizure, day 5	Grade 3, day 15		Local bleeding (catheter track)
12						
13	10			Grade 2, day 60		
14			Partial seizures, days 13 and 23	Grade 2, day 30		
15						
16			Transient fatigue (grade 2)			
17		38.3, day 4		Grade 2, day 15		
18		38.4, day 3	Transient fatigue (grade 2)		Grade 2, day 15	
19	20		Partial seizure, day 0 Transient fatigue (grade 2)			
20		39.3, day 3	Transient fatigue (grade 2) Partial seizure, day 24			
21		38.1, day 3	Partially regressive neurological worsening Partial seizure, day 12	Grade 3, day 60	Grade 3, day 30	Hyponatremia grade 3, day 2 Thrombopenia, grade 2, day 60
22			Transient neurological worsening			
23			Transient neurological worsening			Thrombopenia, grade 2, day 60
24			Transient fatigue (grade 2)	Grade 3, day 30	Grade 3, day 30	

Antitumor Response

All patients but one were assessable for tumor response: Patient 12 wanted to be taken off protocol in order to be treated with temozolomide one month after administration of CpG-28.

Two patients (patients 9 and 17), whose tumors were growing at the time of inclusion, showed minor response (29% and 20% reduction, respectively, in the product of the largest perpendicular diameters) at the injection sites (Figs. 1 and 2). These local responses were associated with reduced mass effect and decreased surrounding edema. Two other patients had stable disease for more than four months (progression-free survival at four months, 9%).

At the time of analysis, 20 patients had died. One-year survival was 28% (Fig. 3). Median survival time for all patients was 7.2 months from time of enrollment (95% confidence interval, 4.8–12.7 months). The progression-free survival at six months was 4.5%.

Discussion

CpG ODNs are exciting new immunostimulating agents that are currently under clinical trials in cancer patients, either as single agents or combined with monoclonal antibodies or chemotherapy. In the present study, we report phase 1 results of a new CpG ODN, administered locally by convection-enhanced delivery in patients with recurrent glioblastoma. This is the first report of CpG ODN in cancer as a stand-alone approach.

Intracranial administration of CpG-28 was generally well tolerated. Given the immunostimulating properties of CpG ODN, the main concern was that CpG-28 might induce local inflammation or trigger an autoimmune disease. Indeed, it was recently reported that intracerebral injection of CpG ODN can induce experimental allergic encephalitis in mice previously immunized against a neural antigen (Conant and Swanborg, 2004). Increased edema or evidence of autoimmune disease was

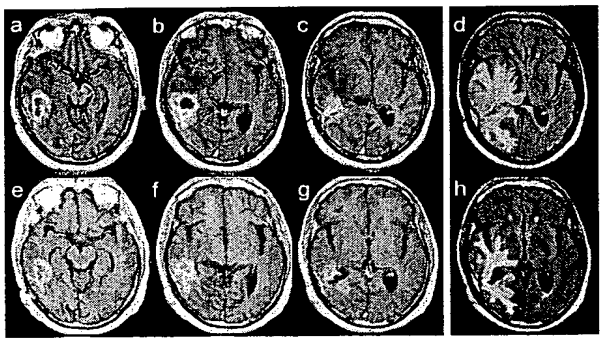

Fig. 1. Brain MRI studies of patient 9. Before administration of CpG oligodeoxynucleotide (ODN), gadolinium-enhanced sections (a–c) and fluid-attenuated inversion recovery (FLAIR) sequence (d) showed a recurrent tumor in the right temporal lobe with surrounding edema. Thirty days after administration of CpG ODN, the area of contrast enhancement, the surrounding edema, and the mass effect were reduced at the site of injection (e–h).

not observed in any of our patients, which suggests that this risk may be minimal. However, we cannot exclude the possibility that our patients were protected by the concomitant use of steroids, and careful monitoring of future patients treated with CpG ODNs is warranted.

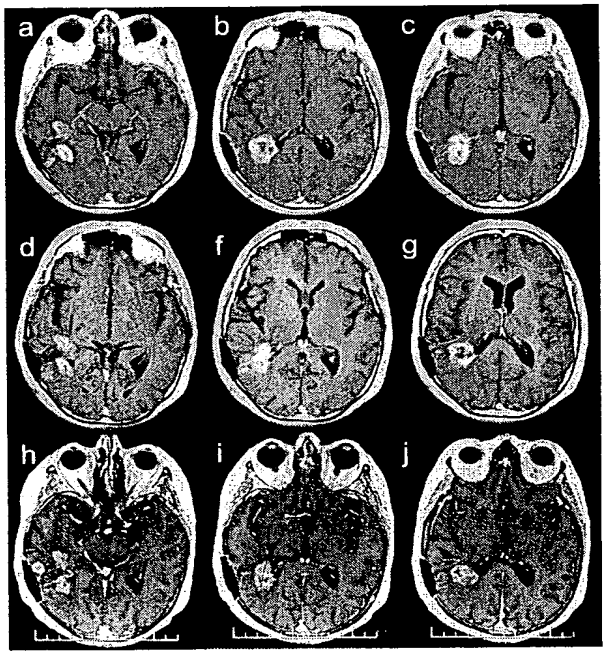

Fig. 2. Brain MRI studies of patient 17. At time of inclusion, gadolinium-enhanced sections (a–c) showed recurrence in the right temporal lobe. MRI performed 30 days (d–f) and 60 (g–i) days after administration showed a minor response at the site of injections. Reduction of surrounding edema was also noted (not shown). A small recurrence occurred on the external part of the temporal lobe (g).

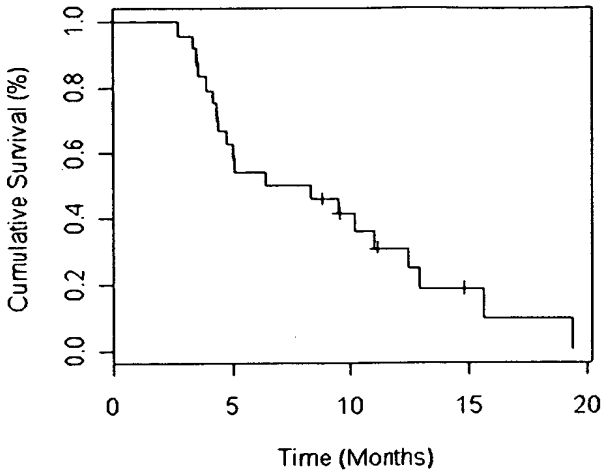

Fig. 3. Kaplan–Meier estimate of overall survival.

Transient neurological worsening was the most significant toxicity observed at the 10- or 20-mg dose level, an adverse event that was not related to increased edema. In organotypic brain cultures, CpG ODNs were shown to be cytotoxic to neurons, through secretion of inflammatory mediators such as nitric oxide and tumor necrosis factor alpha by microglial cells (Iliev et al., 2004). Such inflammatory cytokines might partly explain the neurological worsening seen in some of our patients. However, this worsening regressed spontaneously and was considered to be a potential DLT in only one patient.

Lymphopenia was the most frequent adverse event. This was unexpected, as CpG ODNs stimulate B-lymphocyte proliferation. We did not observe any dose-response relationship, and it remains unclear whether this lymphopenia is related to the studied drug or to previous treatments with chemotherapy. Preliminary analysis did not suggest that a particular subset of lymphocytes was more affected, but this point is currently under study.

Altogether, a DLT was observed in only one patient at the highest dose, and the MTD was therefore not reached. However, as all patients in the 20-mg dose level experienced either neurological worsening or fatigue, the independent scientific committee recommended the dosage of 20 mg for the phase 2 clinical trial.

Efficacy was not the primary objective of this phase 1 trial, conducted on previously heavily treated patients and with escalating doses. Unfortunately, no immunological surrogate markers are available in clinical trials for patients with recurrent glioblastoma who do not undergo surgical resection. Median survival was 7.2 months, and one-year survival was 28%. These figures compare favorably with previous trials using temozolomide, in which median survival was around six months, and one-year survival was less than 15% (Brada et al., 2001; Yung et al., 2000). It should be emphasized that, in those temozolomide trials, patients were treated at the time of first recurrence of glioblastoma, whereas our population for the most part was treated at the time of

second or third recurrence and therefore carried a worse prognosis. A minor radiological response was observed in two patients. This response rate may be underestimated because contrast enhancement might be induced by local immunostimulation triggered by CpG-28 as well as tumor progression.

Further improvements can be suggested for subsequent trials. Convection-enhanced delivery is subject to a number of variables, such as backflow along the catheters or marked heterogeneity of drug distribution within tumor (Vavra et al., 2004). Administration by two or three catheters (instead of one, as used for the first five doses of this trial) or increasing the infusion time might improve CpG ODN distribution, but it should be stressed that complete coverage of the tumor mass is not theoretically needed in a regimen that aims to trigger an immune response. Applying CpG ODN in newly diagnosed glioblastoma might also be a more appropriate setting than in recurrent glioblastoma because the tumor burden would be lower and these patients are likely to have less immunosuppression.

In summary, this study demonstrated that local treatment with CpG ODNs in patients with recurrent glioblastoma is feasible and well tolerated at doses up to 20 mg. A phase 2 trial is currently ongoing.

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A12

Combination immunotherapy with a CpG oligonucleotide (1018 ISS) and rituximab in patients with non-Hodgkin lymphoma: increased interferon-α/β–inducible gene expression, without significant toxicity

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CpG oligodeoxynucleotides (CpG-ODNs) affect innate and adaptive immune responses, including antigen presentation, costimulatory molecule expression, dendritic cell maturation, and induction of cytokines enhancing antibody-dependent cell-mediated cytotoxicity (ADCC). We conducted a phase 1 study evaluating 4 dose levels of a CpG-ODN (1018 ISS) with rituximab in 20 patients with relapsed non-Hodgkin lymphoma (NHL). Patients received CpG once a week for 4 weeks beginning after the second of 4 rituximab infusions. Adverse events were minimal. Quantitative polymerase chain reaction (PCR) measurements of a panel of genes inducible by CpG-ODN and interferons were performed on blood samples collected before and 24 hours after CpG. A dose-related increase was measured in the expression of several interferon–inducible genes after CpG and correlated with serum levels of 2′-5′ oligoadenylate synthetase (OAS), a validated interferon response marker. Genes induced selectively by interferon-γ (IFN-γ) were not significantly induced by CpG. In conclusion, we have defined a set of gene expression markers that provide a sensitive measure of biologic responses of patients to CpG therapy in a dose-related manner. Moreover, all the genes significantly induced by this CpG are regulated by type 1 interferons, providing insight into the dominant immune mechanisms in humans. CpG treatment resulted in no significant toxicity, providing rationale for further testing of this exciting combination immunotherapy approach to NHL. (Blood. 2005;105:489-495)

Introduction

Few advances in the treatment of non-Hodgkin lymphoma (NHL) have had such dramatic impact as the anti-CD20 chimeric monoclonal antibody rituximab (Rituxan; BiogenIdec, Cambridge, MA, and Genentech, South San Francisco, CA).[1] Rituximab compares favorably with single-agent chemotherapy, and has a significantly better toxicity profile, when used in patients with relapsed indolent NHL.[2] However, indolent NHL remains an incurable disease with standard therapy.[3] At least 50% of patients with recurrent indolent NHL do not respond to rituximab therapy, and all patients will experience disease progression at some point after rituximab monotherapy.

The mechanism of cytotoxicity induced by rituximab therapy in humans is not completely understood,[4] and probably includes several interactive mechanisms.[5] Direct apoptosis from cross-linking of CD20 has been observed in some malignant B-cell lines.[6-8] Complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) have been demonstrated in vitro.[9,10] Rituximab in vitro has been shown to translocate CD20 into lipid rafts and to activate complement-mediated lysis;[11] however, there is little convincing evidence that this finding is important therapeutically.[12] In studies of normal and malignant human B cells in vitro, B-cell depletion was observed with rituximab therapy in the presence of mononuclear cells, but not in the presence of complement,[13] suggesting the importance of cell-mediated mechanisms (ADCC). Moreover, the expression of complement inhibitors (CD55 and CD59) on tumor cells and their susceptibility to in vitro complement-mediated killing did not predict clinical outcome after in vivo treatment with rituximab in a recent clinical trial.[14] ADCC, therefore, appears to be a major in vivo mechanism of rituximab.[15] A contribution of ADCC is supported by a clear role for the FcγR-bearing effectors in mediating response to rituximab in certain lymphoma histologies.[16,17]

Immunostimulatory sequences (ISSs) are short sequences of synthetic DNA containing unmethylated CpG dimers that have multiple effects on the human immune system.[18] ISSs signal through the Toll-like receptor 9, which is expressed on only a few specific subsets of immune cells.[19] Immunostimulatory effects of CpG-ODN include the induction of proliferation and immunoglobulin production by B cells and the induction of interferon-α (IFN-α), IFN-β, interleukin-12 (IL-12), and tumor necrosis factor α (TNF-α) by plasmacytoid dendritic cells (DCs). These cytokines, in turn,

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produce potent responses in many other immune cell types not directly responsive to CpG-ODN. IL-12, TNF-α, and, especially, IFN-α activate cytotoxicity by natural killer (NK) cells, and IL-12 induces strong NK production of IFN-γ.[20] This cytokine milieu induces the differentiation of naive $CD4^+$ T cells into T-helper 1 (T_H1) cells on encountering specific antigens. Moreover, CpG-ODN increases antigen presentation and costimulatory molecule expression.[21] CpG-ODN specifically affects the maturation of DCs, which are recognized as distinct antigen-presenting cells (APCs) largely responsible for the initiation of T-cell and NK-cell responses to tumor antigens.[22-25] Synthetic CpG-ODN sequences (ISSs) are under investigation for a number of therapeutic indications—including protection from infectious pathogens, adjuvant for vaccines, and treatment of allergy—by encouraging T_H1 rather than T_H2 immunity.[26]

We hypothesized that an ISS could have significant synergistic effects with antitumor monoclonal antibodies such as rituximab through the augmentation of ADCC and the enhancement of presentation of antigens released by killed tumor cells. Therefore, we designed a phase 1 study testing 4 doses of a CpG-ODN (1018 ISS; Dynavax Technologies, Berkeley, CA) with rituximab in patients with relapsed or refractory advanced-stage NHL. In the results reported in this article, the combination was well tolerated and demonstrated significant biologic activity, measured through a dose-related increase in type 1 interferon–inducible genes. Unlike therapy with exogenous interferon or interleukins, the 1018 ISS and rituximab combination resulted in no significant toxicity, providing rationale for further testing of this exciting combination immunotherapy approach to NHL.

Patients, materials, and methods

Informed consent was obtained in writing from all patients before enrollment. This study was approved by the Institutional Review Board at Dana-Farber/Partners Cancer Care and abides by the tenets of the Declaration of Helsinki.

Patients

Eligible patients had histologically documented $CD20^+$, B-cell NHL as defined by the World Health Organization (WHO) classification, and received at least 1 previous treatment regimen for lymphoma. All patients were 18 years of age or older and had a performance status using the Eastern Cooperative Oncology Group (ECOG) score of 2. Eligible patients were required to have white blood cell (WBC) counts greater than 2000 (1000/μL), absolute neutrophil counts (ANCs) greater than 1000 (1000/μL), platelet counts greater than 75 000/mm^3, and expected survival times greater than 4 months.

Patients not eligible to be enrolled in the study included those who were pregnant or lactating or who used chemotherapy, systemic steroids, or radiation within 30 days before study enrollment. Additionally, patients treated with radioimmunotherapy (including iodine I 131 tositumomab or ibritumomab tiuxetan), autologous transplantation, or fludarabine chemotherapy *within the previous 6 months* were excluded because of the immunosuppressive nature of these therapies. Patients experiencing disease progression, as defined by the National Cancer Institute (NCI)–Sponsored International Working Group criteria[27] within 6 months of any previous rituximab-containing therapy were also excluded. Other exclusion criteria included history of allogeneic (bone marrow or stem cell) transplantation, including nonmyeloablative transplantation; history of unstable angina, symptomatic cardiac arrhythmia, or clinical heart failure; severe pulmonary disease, symptomatic pleural effusions, or clinically significant pulmonary symptoms; uncontrolled bacterial, viral, or fungal infection; clinically apparent central nervous system (CNS) lymphoma; major surgery within 2 weeks before enrollment; known presence of human antimurine antibodies (HAMAs) or antichimeric antibodies (HACAs), or history of any clinically significant autoimmune disorder. Because other phosphorothioate compounds interfere with the interpretation of coagulation assays in vitro,[28] an additional exclusion criterion was current therapeutic use of anticoagulants or history of symptomatic coagulopathy.

Treatment

Rituximab was given on days 1, 8, 15, and 22 (weekly × 4 doses) at a dose of 375 mg/m^2, as previously described.[29] Between 30 and 60 minutes before the start of the rituximab infusion, the patient was given oral acetaminophen (650 mg) and diphenhydramine hydrochloride (50 mg). Corticosteroids were avoided.

The CpG oligonucleotide 1018 ISS (provided by Dynavax Technologies) is a single-stranded, 22–base pair (bp) immunostimulatory phosphorothioate oligonucleotide prepared by standard solid-phase chemistry techniques (sequence 5′-TGACTGTGAACGTTCGAGATGA-3′) with a molecular mass of approximately 7150 Da.[30] Patients were assigned to 1 of 4 doses of 1018 ISS: 0.01 mg/kg, 0.05 mg/kg, 0.2 mg/kg, or 0.5 mg/kg. Dose assignments were sequential and were based on the order in which patients consented and completed the screening process. Dose assignments began with the lowest dose, and the next cohort at the next higher dose began treatment only after investigators reviewed the safety parameters of the preceding dose cohort.

The first injection of 1018 ISS was administered subcutaneously 30 to 60 minutes after the second dose of rituximab (on day 8). The second and third doses of 1018 ISS were administered 30 to 60 minutes after the third and fourth infusions of rituximab. The fourth (last) dose of 1018 ISS was administered 1 week after the fourth (last) dose of rituximab, on day 29.

Dose-limiting toxicity and rules for stopping

A classical phase 1 design was used for this study. Dose-limiting toxicities (DLTs) (defined by the NCI Common Toxicity Criteria, version 2, http://ctep.cancer.gov/forms/CTCv2.0_4-30-992.pdf) were grade 2 or higher allergic reaction to 1018 ISS and any nonhematologic toxicity grade 3 or higher that occurred during the 1018 ISS treatment period, and that was deemed possibly or probably related to 1018 ISS treatment. Hematologic toxicities were defined in this study as any episode of febrile neutropenia, platelet count below 20 000, and platelet transfusion for bleeding.

Each dose cohort consisted of 3 patients who completed both rituximab and 1018 ISS treatments. Patients withdrawn because of toxicity counted as having had a DLT. Patients who withdrew for any reason other than toxicity were replaced. If no patient had a DLT after all 3 patients in a cohort completed therapy, enrollment of the next cohort commenced. If 1 patient had a DLT, 3 additional patients were enrolled at that dose level; if none of these 3 additional patients had a DLT, then enrollment at the next dose level commenced. If 2 or more patients had DLTs, then the previous dose level was declared the maximum tolerated dose (MTD). At the conclusion of dose escalation, 6 additional patients were entered in the candidate MTD tier; if 1 or fewer DLTs were observed in these 6 additional patients, the candidate MTD was declared the MTD; otherwise, de-escalation to the next lower dose occurred, and 6 additional patients were added at that dose.

Response criteria and follow-up

Clinical responses were as previously defined by the International Workshop for NHL response criteria.[27] Patients underwent comprehensive restaging, including physical examination and anatomic imaging, on day 56 of therapy, then every 3 months for the first year, then every 6 months, until evidence of disease progression.

RNA preparation and quantitative gene expression analysis of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated using Vacutainer CPT tubes (BD Biosciences, San Jose, CA), and duplicate samples of 1×10^6 cells were resuspended in RLT-buffer (Qiagen, Valencia, CA) and subsequently were stored at −80°C. From each sample, RNA was purified

using RNeasy mini kits (Qiagen). The RNA was DNase treated and reverse transcribed, and subsequently cDNA levels were quantitated by real-time polymerase chain reaction (PCR) using a Perkin-Elmer 5700 Sequence Detection System (all methods as previously described).[31] The intercalating dye SYBR green (Qiagen) or a specific probe labeled with a fluorescent dye (Perkin-Elmer) was used to detect the PCR product at each cycle. Specific PCR primer/probe pairs were obtained from Perkin-Elmer or were designed and validated by us. When using SYBR green, the specificity of each reaction was confirmed by a melting temperature curve at the end of each run. Results were calculated as a ratio of the test gene to the internal control gene ubiquitin. This gene/ubiquitin ratio is directly proportional to the fraction of total mRNA represented by the test gene and normalizes for experimental variations in RNA recovery, purification, and quality. We have confirmed that the expression of ubiquitin is linear over a broad range of RNA concentrations by processing, under identical conditions, human PBMC samples ranging from 5×10^3-10^7 cells. The standard deviation of the gene/ubiquitin ratio in replicate samples is generally less than 50% of the mean of the replicates.

Serum markers of ISS activity

Because the induction of interferon is an expected effect of ISS, we incorporated the evaluation of validated interferon-induced markers that are dose-dependent indicators of serum interferon levels.[32] These included evaluation serum 2′5′ oligoadenylate synthetase (OAS), which is inducible in monocytes and lymphocytes by IFN-α and IFN-β and has been widely used to monitor pharmacokinetics in phase 1 trials of type 1 interferon.[33] In addition, serum levels of neopterin, which is produced by the interferon-inducible enzyme GTP cyclohydrolase and is secreted by macrophages in response to interferons, were determined. Both 2′5′ OAS and neopterin are at least as sensitive as enzyme-linked immunosorbent assay (ELISA) detection of serum interferon.[34]

Biostatistical analysis

A standard phase 1 design was selected for this study. The primary end point was to determine safety and tolerability of 1018 ISS in conjunction with rituximab, as determined by type and severity of adverse events and by clinically significant changes in laboratory results, and to determine the MTD for 1018 ISS given after rituximab infusions. Secondary exploratory variables included clinical response rate and biologic activity of 1018 ISS in conjunction with rituximab. Gene expression data were evaluated by Kruskal-Wallis nonparametric analysis of variance (ANOVA) with Dunn posttest to compare differences between groups.

Results

Patients

Twenty patients (11 women, 9 men) were enrolled; the median age was 59 (range, 40-73 years). Histology included follicular NHL in 17 patients, diffuse large B-cell NHL in 2 patients, and small lymphocytic lymphoma in 1 patient. Patients had received a median of 3 previous therapies for NHL, detailed in Table 1. Of note, 4 patients were treated with aggressive salvage chemotherapy, 2 underwent autologous stem cell transplantation, 7 received rituximab, 1 received previous radioimmunotherapy, and 4 underwent previous external beam radiation therapy.

Median time from diagnosis to enrollment was 36 months (range, 12 to 145 months). Bone marrow involvement was present histologically at enrollment in 5 of 20 patients who underwent evaluable bone marrow biopsy. Two patients had B symptoms. One patient did not achieve remission. The remainder of the patients had disease progression after response to previous therapy.

Table 1. Patient characteristics

Characteristics	No.
Total enrolled	20
Women/men	11/9
Mean age, y (range)	59 (40-73)
Histology	
Follicular NHL grade 1*	15
Follicular NHL grade 2*	2
Diffuse large B cell NHL*	2
Small lymphocytic NHL*	1
Previous therapy	
CHOP	9
CVP	9
Rituximab	7
Oral alkylating agents	4
Purine analog chemotherapy	3
Other combination chemotherapy	4
Autologous stem cell transplantation	2
Ibritumomab tiuxetan	1

CHOP indicates cyclophosphamide, hydroxydaunomycin/doxorubicin, Oncovin, prednisone; CVP, cyclophosphamide, vincristine, prednisone.
*World Health Organization lymphoma classification.

Treatment

Eighteen patients completed 4 infusions of rituximab and the 4 prescribed doses of 1018 ISS. One patient discontinued protocol participation after the third infusion of rituximab because of the exacerbation of underlying chronic back pain, unrelated to disease status or protocol therapy. One additional patient discontinued protocol participation before receiving any ISS therapy.

Adverse events and laboratory changes

Fifty percent of patients had expected infusion reactions associated with the initial dose of rituximab; there was no exacerbation of rituximab toxicity after the institution of ISS therapy. Common adverse events observed in this study are detailed in Table 2. Injection site reactions occurred in a dose-dependent fashion. The only grade 3 adverse events included the aforementioned back pain, an episode of sepsis thought unrelated to therapy, an episode of atypical pneumonia thought possibly related to therapy, and an episode of confusion caused by narcotic use for pain secondary to NHL. No patient developed HAMA after combination therapy. Antinuclear antibody (ANA) levels did not significantly change compared with baseline levels.

Response to 1018 ISS measured by interferon-induced gene expression

We used quantitative PCR analysis to evaluate changes in mRNA expression in a panel of interferon-inducible genes between PBMCs isolated before and 24 hours after the second and fourth doses of 1018 ISS. These genes are detailed in Table 3. Gene expression levels in baseline PBMC samples, collected on 2 visits before the start of treatment, varied 10- to 100-fold among patients ($n = 19$) but were relatively stable within individual patients ($r = 0.978$; $P < .001$). There was no evidence of gene induction in vivo by the 0.01 mg/kg dose, but a dose-related increase was observed in the induction of several IFN-α/β–inducible genes 24 hours after the injection of 1018 ISS in the 3 higher-dose groups. Gene expression data from a representative patient in the second cohort is depicted in Figure 1. Specifically, 2 of 4 patients in cohort 2 (0.05 mg/kg) showed greater 3-fold induction of 2 or more IFN-inducible genes. In cohorts 3 and 4 (0.20 mg/kg and 0.50

Table 2. Frequent and serious adverse events reported during treatment period

Event	Grade 1	Grade 2	Grades 3-4	Total, %
Allergic reaction*	5	5	0	50
Injection site reaction†	6	3	0	45
Upper respiratory‡	7	1	0	40
Headache	6	2	0	40
Fatigue	2	4	0	30
Pneumonia	0	0	2	10
Confusion	0	0	1	5
Back pain	0	0	1	5
Sepsis	0	0	1	5

Values reflect events that occurred in more than 20% of patients and all grades 3-4 events. No grade 5 events were reported.

*Includes hives, rhinitis, rigors, chills, and dyspnea.

†Injection site reactions were dose-dependent.

‡Includes cough, congestion, infection.

mg/kg), all patients showed greater than 3-fold induction in at least 2 of these genes. Moreover, the mean induction levels of most of these genes tended to increase with increasing doses. There was no significant difference in biologic response between cohorts 3 and 4, suggesting we were reaching a plateau in biological activity. This dose-related gene induction was only observed, however, in genes inducible with type 1 interferons or inducible equally well with types 1 and 2 interferons. The 2 genes induced selectively by IFN-γ *(MIG, IRF-1)* were not significantly increased by 1018 ISS at any dose level. Figure 2 summarizes gene-induction data for all patients, demonstrating this selective type-1 interferon effect.

Interferon-induced serum markers

There was no significant increase in serum levels of neopterin after therapy with ISS (data not shown). In cohorts 1 and 2, there were no significant increases in serum OAS concentration. However, 6 of 9 patients with adequate samples in cohorts 3 and 4 demonstrated an increase in OAS after ISS therapy compared with baseline. Five of these 6 patients also demonstrated increases in *OAS* gene expression as measured by quantitative PCR.

Clinical response and patient outcome

Nineteen patients were evaluable for response. Six patients showed response (1 unconfirmed complete remission [CRu], 5 partial remission [PR]) for an overall response rate of 32% (90% confidence interval, 17%-64%; computed using logarithmic transformation). Additionally, 13 patients had stable disease after therapy. Median progression-free survival in responding patients is 12 months (range, 5-23.5 months). Four patients remain alive without progression at a median of 10 months follow-up (range, 3.2 to 23.4 months). Four patients have died: 1 of progressive disease, 1 of complications of chronic obstructive pulmonary disease (COPD) with lymphoma present at death, 1 of sepsis after additional chemotherapy, and 1 of a pulmonary embolism without evidence of lymphoma at the time of death.

Table 3. Biologic response marker genes for IFN and ISS activity

Symbol	Name	Induced in vitro		Induced in patients
		IFN-α	IFN-γ	ISS
2′-5′ OAS	2′-5- Oligoadenylate synthetase	+	−	+
ISG-54	Interferon-stimulated gene, 54 kDa	+	−	+
MxB	Myxoma resistance-B	+	−	+
MCP-1	Monocyte chemotactic protein	+	+	+
MCP-2	Monocyte chemotactic protein	+	+	+
IP-10	IFN-γ–inducible protein 10	+	+	+
IRF-1	Interferon-responsive factor-1	−	+	−
MIG	Monokine induced by IFN-γ	−	+	−

+ Indicates that gene was induced.

− Indicates that gene was not induced.

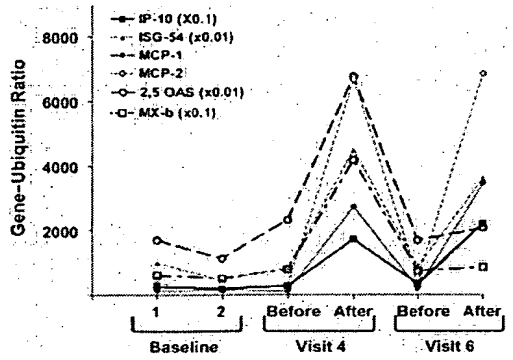

Figure 1. Gene induction by 1018 ISS in a representative patient. Gene/ubiquitin ratios for 6 interferon-inducible genes in PBMCs from patient 113, treated with the highest dose of 1018 ISS (0.5 mg/kg). Values for the more highly expressed genes were multiplied as indicated. Samples were taken before and 24 hours after 1018 ISS injection. Visit 4 included the second and visit 6 included the last of the 4 injections of 1018 ISS.

Discussion

Our study represents the first published experience of a CpG ODN in combination with an antitumor monoclonal antibody. In our study, 1018 ISS treatment was extremely well tolerated, at weekly doses of up to 0.5 mg/kg, without the toxicities usually associated with pharmacologic doses of cytokines. This dose is approximately 10-fold higher than that used in previous studies of injected 1018 ISS as a vaccine adjuvant.[35] Moreover, the demonstration of significant dose-related increases in ISS-inducible genes demonstrates that this study encompassed a range of 1018 ISS doses with substantial pharmacologic activity in humans.

Many of the therapeutic actions of ISS are likely mediated through the induction of IFN-α and IFN-β from ISS-responsive plasmacytoid dendritic cells and IFN-γ indirectly from NK cells.[26] Genes prominently induced by interferons have been extensively characterized, and several gene products have been used to monitor clinical trials of interferons. Interferon-inducible mRNAs, however, have not been widely used in the same manner, though a recent study in patients with hepatitis C virus (HCV) used microarrays to measure changes in the mRNA of PBMCs 3 and 6 hours after IFN-α therapy.[36] For our study, we selected 8 genes with known induction patterns and, in some cases, well-studied regulatory regions. In preliminary experiments with cultured human PBMCs (data not shown), these genes were shown to be strongly induced by 1018 ISS and IFN-α or IFN-γ, or both. Although the kinetics of expression of these genes varied, mRNA levels remained substantially elevated after 24 hours for all genes. In contrast, IFN-α and IFN-γ mRNAs were transient and variable, and mRNA levels were relatively low. Thus, the 24-hour time point was chosen for evaluation of posttreatment blood samples to assess ISS activity.

Genes regulated by IFN-α, or by either type of interferon, were induced in a dose-dependent fashion. The 2 genes regulated

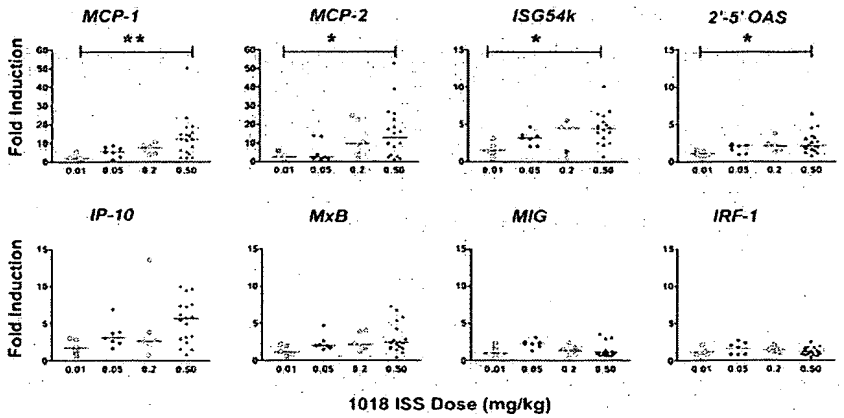

Figure 2. Summary of interferon-inducible gene response to injection of 1018 ISS. Data are expressed as fold induction after 1018 ISS compared with the matching pre–1018 ISS value. Significance of the differences among groups was analyzed by the Kruskal-Wallis test, with the following values: *MCP-1*, $P = .005$; *MCP-2*, $P = .016$; *ISG54k*, $P = .024$; *2'-5' OAS*, $P = .033$; all other genes, $P > .05$. Comparisons of the 0.01 and the 0.5 mg/kg groups by Dunn posttest are indicated, if significant, on the figure. $^{**}P < .01$; $^{*}P < .05$.

primarily by IFN-γ, however, showed no induction, suggesting that much of the biologic activity of 1018 ISS in vivo is mediated by type-1 interferons. This was surprising because 1018 ISS is representative of the CpG-B class of ISS and induces low, often undetectable levels of IFN-α in cultured PBMCs in vitro.[37,38] Other CpG-ODN sequences, including those under clinical evaluation, may have different effects.[39] Clearly, though, our studies provide evidence that 1018 ISS administered subcutaneously has a biologically relevant, systemic effect in patients with lymphoma treated with an ISS/antibody combination.

CpG-ODNs have been shown in animal models to significantly enhance monoclonal antibody therapy against malignancy in vivo. In one study of anti-idiotype monoclonal antibody treatment of murine B-cell lymphoma, ISS alone had no effect on the survival of mice inoculated with 38C13 murine lymphoma cells. However, a single injection of an ISS significantly enhanced the antitumor response to monoclonal antibody therapy: 90% of mice treated with monoclonal antibody alone developed lymphoma compared with only 20% of mice treated with antibody and ISS.[40] This combination was as effective as multiple doses of IL-2 at inhibiting tumor growth when combined with monoclonal antibody therapy, with significantly less toxicity. Further studies using the 38C13 tumor model suggest that efficacy of monoclonal antibodies is most enhanced when CpG-ODN is given within 2 days of antibody therapy.[41] Furthermore, in indolent lymphoma cell lines, CD20 expression, in particular, has been observed to increase in response to CpG-ODN.[42,43] Interestingly, an inverse correlation was detected between baseline expression of CD20 and expression after exposure to CpG-ODN; the most significant increases in CD20 expression were found in samples that had the lowest baseline levels, suggesting another possible mechanism through which 1018 ISS could augment responses to anti-CD20 therapy with rituximab.

A murine study provides further insight into the mechanism of possible antitumor effects of CpG-ODN in NHL.[44] Mice receiving CpG-ODN had significant suppression of tumor growth after subcutaneous injection with EL4 cells, with the greatest effect seen in mice given CpG DNA near the tumor inoculation site. NK cells, monocytes, and macrophages were necessary for this antitumor effect. Mice that received CpG-ODN rejected subsequent tumor rechallenge, and further studies support a T-cell memory response. Another murine study demonstrated significant enhancement of T_H1 immunity through IL-12 production and HLA class 1 and 2 molecules when CpG-ODN was combined with an antigen/granulocyte macrophage–colony-stimulating factor (GM-CSF) fusion protein.[45] Finally, a recent study reveals that daily injection of CpG-ODN dramatically alters the morphology and functionality of mouse lymphoid organs, potentially altering the lymphoma microenvironment.[46]

Because ADCC appears to play an important role in the response to rituximab and other monoclonal antibodies, several alternative approaches to stimulate effector-cell function in vivo have been used to augment the clinical response. Several years ago, Vlasveld et al[47] treated 7 patients with indolent B-cell lymphoma with a combination of a murine anti-CD19 antibody and continuous infusion low-dose IL-2, a lymphokine that enhances ADCC in vitro. A gradual and sustained increase in $CD8^+$ and $CD56^+$ cells occurred, and 2 responses were observed. Lymphocytes from involved lymph nodes also showed enhanced ADCC, which provided proof-of-principle. Others[48] and we[29] have published results of clinical trials evaluating systemic IL-2 combined with rituximab in patients with NHL, demonstrating relative safety and effector-cell enhancement and suggesting a prolonged time to progression in a subset of patients with follicular NHL. Rituximab has also been safely combined with IL-12,[49] another cytokine that mediates effector cell number and function; G-CSF,[50] which greatly enhances the cytotoxicity of neutrophils in ADCC; and IFN-α,[51] an immunomodulatory cytokine that induces antigen expression, enhances cytotoxicity of immunotoxins, and has limited clinical activity as a single agent for NHL.[52] Additional clinical trials of these combinations are ongoing;[53] however, the doses of cytokines required to achieve adequate effector-cell responses are associated with greater toxicity than those of rituximab alone.[54]

Recently, a phase 1 study of a similar ISS (CpG 7909; Coley Pharmaceutical Group, Wellesley, MA) was completed in patients with previously treated NHL.[55] In 15 patients evaluable for effector cell changes, dose-related increased ADCC activity and increased NK-cell activity were observed at day 21 compared with baseline levels. The regimen was easily tolerated, and, despite no clinical responses, the significant immunomodulatory activity suggested potential for benefit in combination with standard treatment approaches. One potential concern in using CpG-ODN for lymphoma is that CpG-containing sequences, including 1018 ISS, have been observed to induce proliferation of primary human B cells in vitro.[56-59] Lymphoma proliferation was not observed in vivo in the single-agent study by Link et al[55] in patients with relapsed NHL or in our study. Moreover, in the EG7/EL4 murine lymphoma model, CpG-ODN treatment resulted in a significant suppression of tumor growth.[44]

Given the favorable safety and tolerability profile to date in humans, including our current study, 1018 ISS appears to be an

ideal agent for combination immunotherapy with rituximab. The use of 1018 ISS and rituximab clearly has the potential to enhance ADCC and to induce the antigen-presenting function of DCs, macrophages (MFs), and B cells. Subsequently, activated APC may induce T_H1 and cytolytic T-cell expansion, thereby generating tumor-specific immune responses. We have developed a phase 2 study to evaluate the efficacy of 1018 ISS combined with rituximab in patients with relapsed follicular NHL, using the 0.2 mg/kg dose, the lowest dose observed that provided the maximum biologic effects. In this trial, we plan to further explore the effects of this rational combination on the tumor microenvironment and systemic immune function through surrogate markers of biologic activity. Additionally, we will correlate these findings, including the gene expression data, with clinical responses. Through detailed analysis of these surrogate markers, we hope to better define the optimal schedule of this combination, which may require additional phase 2 studies. Other combination immunotherapy trials, using different ISSs, are also ongoing.[60] We anticipate that these CpG-ODN combinations with rituximab will have very low toxicity in this patient population, which is a critical part of developing novel therapeutics for patients with indolent NHL.

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